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PHYSIOLOGICAL REVIEWS

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THE AMERICAN PHYSIOLOGICAL SOCIETY

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VOL. 15, No. 2

Issued April, 1935

BALTIMORE, U. S. A.

Entered as second-class matter, January 15, 1921, at the Post Office at Baltimore, Md., under the act of March 3, 1879

Made in United States of America

PHYSIOLOGICAL REVIEWS

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Subscriptions will be received in advance only. The subscription price is:

$6.00 per volume in the United States, net postpaid
$6.25 per volume in Canada, net postpaid
$6.50 per volume elsewhere, net postpaid
$2.50 per single number, net postpaid

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PHYSIOLOGICAL REVIEWS

VOL. 15 APRIL, 1935 No. 2

MANGANESE: ITS DISTRIBUTION, PHARMACOLOGY AND HEALTH HAZARDS

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DISTRIBUTION OF MANGANESE. Manganese occurs as the oxide in the following minerals; Pyrolusite (MnO_2), Braunite ($Mn_2O_3, 3Mn_2O_3 \cdot MnSiO_3$), Manganite (MnOOH), Hausmannite (Mn_3O_4), Franklinite ($FeZnMn(FeMn)_2O_4$); as sulfide in Manganeseblende (MnS) and Hauserite (MnS_2); as carbonate in Manganesespat ($MnCO_3$); as silicate in Tephroite (Mn_2SiO_4), Knebelite ($(FeMn)_2SiO_4$), Heterocline, Helvine, and Rhodonite ($MnSiO_3$ or $MnOSiO_2$). It is characterized by the great number of different oxygen compounds which it may form, of which the following have been described: manganous oxide (MnO), manganous oxide-oxide (Mn_3O_4), manganese sesquioxide (Mn_2O_3), manganese peroxide (MnO_2), manganese trioxide (MnO_3), manganese heptoxide (Mn_2O_7) and manganese tetroxide (MnO_4). It forms two types of salts, of which the manganous salts are derivatives of the bivalent and the manganic salts are derivatives of the trivalent manganese. Manganic acid is a derivative of mangan trioxide and permanganic acid of mangan heptoxide.

Manganese was discovered by Scheele (1774) in pyrolusite and isolated in pure form by Gahn (1807). Scheele found it also in the soil; Leclerc (1872) confirmed this for a great number of soils from different localities.

OCCURRENCE OF MANGANESE IN PLANTS. Manganese appears to be a constant constituent of plants. Von Schroeder (1878) found it in the ashes of pine needles and of pine bark. Its presence in a great variety of plants was established by Maumené (1884) who also found it present in a number of red and white wines to the extent of 0.0001 to 0.0020 gram per liter. Headden (1915) found it in wheat regardless of the type of soil and climate. He found that the wheat kernels contained about

equal quantities of manganese and iron. Bertrand and Rosenblatt (1921) extended the studies of Maumené and found it also in those plants in which the latter was unable to discover it. Pichard (1898) had assumed that manganese is concentrated in those parts of the plant which are in active vegetation and also in very young shoots, and Jardin and Astruc (1913) found that the chlorophyll-containing parts are richer in manganese than the subterranean parts. Bertrand and Rosenblatt (1922) determined the manganese content of different structures of Nicotiana rustica L. and of Lilium lancifolium rubrum and found more manganese in those organs in which chemical reactions (transformations) take place. The reproductive organs, calyx and stamen contained 42.7 and 40.9 mgm. per 100 grams of the ash. They found the wood which has only very little reproductive activity remarkably poor in manganese (4.8 to 6.4 mgm. per 100 grams of ash). The seeds contained 127.7 mgm. per 100 grams of ash. Wester (1921) had made an extensive study of the manganese content of different seeds and had found considerable variations. McHargue (1924) pointed out that with cereals the greater part of the manganese is lost in the polishing and milling. From the work of Czapek (1925) (quoted from Richards) it appears that during the process of ripening the absolute values of manganese in the seeds shows an increase, he quotes that Arendt (1860) reported similar results for Avena sativa and Schjerning (1906) observed the same phenomenon for ripening barley. Bertrand and Rosenblatt also found that young leaves usually contain more manganese than older ones. Bishop (1928) reported similarly that buds, petioles, leaves and twigs contained most, roots less, and small branches, large branches and trunk timber least. In flowers the manganese content of the different organs is decreased in the following order: ovaries, styles, and stigmata, anthers, perianth and peduncles. It appears, therefore, that the reproductive parts have the highest manganese content and the woody sections contain very little. Richards (1930) showed that there is a wide difference in the manganese content of the reproductive organs of different plants even of the same species and assumed that this depends largely upon the abundance of the water supply and its manganese content. This is supported by the statement of Kobert (1883) that true water plants, such as Zostera marina and Trapa natans have a very high percentage of manganese. Bishop assumed that there must be some relation between the manganese content and the formation of chlorophyll and hence with the assimilation of carbon in plants. Richards (1930) determined the manganese content of a great variety of foodstuffs.

It appears, therefore, that manganese is a constant constituent of plants and that it may have some important functions in their metabolism.

Scheele (1774) had noticed that the manganese of the soil is assimilated by plants. Aso (1902) (quoted from Bishop) found that manganese in small concentrations has a favorable effect on the growth, whereas high concentrations develop toxic effects. Voelker (1903) experimented with manganese salts as fertilizers in field experiments. He found that the chloride and nitrate produce good color in plants and that the iodide retarded distinctly the germination and the growth. Manganese chloride, phosphate and sulfate and also red manganoxide (Mn_3O_4) increased the yield. Bartmann (1910) found manganese dioxide less effective than manganese sulfate in promoting growth of plants. Bertrand (1905) also found that fertilization with manganese increased the yields of the crops and that the individual grains were heavier than those of the control fields. Similar results were also reported by Katayama (1906) and Brenchley (1910), but they noted that larger quantities cause bleaching of the plants. The use of manganese as a fertilizer has been reviewed by Bocher (1922). Toxic effects were observed by Loew and Sawa (1903), Kelley (1909), Guthrie and Cohen (1910), Willcox and Kelley (1912), Skinner and Sullivan (1914) and Deatrick (1916) (quoted from Bishop) and before these Sachs (1865), later Birner and Lucanus (1866) and Wagner (1871) had stated that an excess of manganous and manganic phosphate had an injurious effect on plants.

McHargue (1923) found leguminous plants much more sensitive to the lack of manganese than non-legumes, although only very small quantities of manganese are required for the normal growth of plants. Seeds, which, as demonstrated by Bertrand and others, are especially rich in manganese, do not contain enough to secure normal growth to maturity of the plant; some seeds, however, may have a sufficient amount to maintain a normal development during the first five or six weeks. Several mechanisms for the action of manganese on the growth of plants have been suggested. Hall (1907) postulated an effect of manganese on the soil bases rather than a direct action on the plants. Bernardini (1910) assumed that manganese had some catalytic action on the soil increasing the oxygen absorption. Brown and Miges (1916) claimed that the action of manganese salts consists mainly in their effect on ammonification and nitrification of the soil. The assumption of Johnson (1924) that the toxic effect of manganese is due to a decrease of the assimilation of iron, could not be confirmed by Bishop (1928).

McHargue (1923) found that the toxicity of manganese for plants depends in part upon the acidity of the soil. He reported that the addition of manganese salts to acid soils causes a decrease of the crops; in the same quantity, however, added to an acid soil after its neutralization by means of calcium carbonate, it causes an increase of the yield. He assumed that the toxicity of acid soils may, at least in part, be due to the formation of soluble manganese salts. Bishop found that the addition of calcium carbonate to soils containing manganese salts had an antitoxic effect towards higher concentrations of manganese.

Some of the experiments mentioned indicate that manganese may be involved in the oxygen metabolism of the plant. Bertrand (1897) found that the activity of laccase was increased by manganese salts, the manganese acting presumably as a co-ferment. Aso (1902) observed that the intensity of the color reaction of oxidizing enzymes of plants fertilized with manganese exceeded that of the controls, and Loew and Sawa (1903) found that the juice of plants bleached by an excess of manganese in the soil showed a stronger oxidase and peroxidase reaction than normal plants.

It appears, therefore, that manganese increases the oxidase reaction in plants.

Effect of manganese on tissue activity in animals. Walbum and Moerch (1923) found that the intravenous injection of manganese in goats immunized against diphtheria toxin stimulates the antitoxin formation. In horses they produced an increase of the antitoxin titer even if it was already on the decline. They found oral administration ineffective in this respect and assumed direct stimulation of the antitoxin producing cell mechanism. Later Walbum and Schmidt (1925) found that the injected material disappears rapidly from the circulation and that it is mainly stored in the liver, and they assume that the faculty of this organ to retain manganese is somehow related to the effect on the increased antitoxin formation. Preti (1909) studied the effect of manganese chloride, sulfate, acetate and lactate on autolysis of the liver. When added in small quantities to liver pulp they cause an increase of the noncoaguable nitrogen during autolysis, which progresses with the concentration of the manganese salts. The acetate, however, causes such an increase only within a certain range, larger doses having an inhibiting effect. Bradley and Morse (1915) found with manganese chloride that the stimulation occurs only until a concentration of 1/10 mol is reached, and that further increase of the concentration has an inhibiting effect. They assumed that manganese alters the proteins

in such a way that they become more digestible to the protease. Sato (1929) found that very small doses of manganese stimulate the respiration of tissues, whereas larger doses have a depressant effect.

It appears, therefore, that manganese within certain concentrations stimulates the antitoxin formation, the autolysis of the liver and the respiration of animal tissues.

OCCURRENCE OF MANGANESE IN ANIMAL TISSUES. Marchessaux (1844) and Millon (1847) discovered manganese in the blood. Burin de Buisson (1852) (quoted from Kobert) claimed that manganese is a regular constituent of the blood and suggested that in chlorosis the manganese content of the red blood cells must be increased. But Garrod showed that such an increase is impossible and also Buchheim (1878) was unable to increase the manganese content of erythrocytes of the dog. Glénard (1854) found manganese in the blood of only one person out of forty, and Campani (1872) showed it in the serum and the cells of ox blood. Griffiths (1892) found that the blood of Pinna squamosa (a mollusc) contains as much as 0.19 gram per cent of the ash. Bertrand and Medigreceanu (1912) determined the manganese content of the blood of man, horse, cattle, pig, rabbit, seal, chicken and canary and found from 0.00 to 0.02 mgm. per 1000 cc. of blood, the blood of sheep contained slightly more, namely, 0.06 mgm. per liter. The plasma contained about twice as much as the cells. Reiman and Minot (1920) determined the manganese in the blood of patients suffering from various diseases as 0.004 to 0.025 mgm. per 100 gram, averaging 0.012 mgm. The individual level in the blood was quite constant with repeated examinations. The difference between these figures and those found by Bertrand may be partly due to the improved method of determination used by Reiman and Minot, and partly to the different manganese content of the drinking waters in eastern Massachusetts (0.001 to 0.0015 part per 100,000[3] parts of water). In workers exposed to dust of manganese ores they found 0.013 mgm. per 100 grams of blood which is practically the same as in normal persons.

Manganese appears to be a normal constituent of practically all tissues. Kobert stated that Wurzer (1833) found manganese in human teeth, Weidenbusch and Lehmann (1830) in bile, and Rey, Wurzer and Cottereau (1849) found it in gallstones. Pollacci (1870) detected it in human milk. McHargue (1924) found 5 times as much manganese in colostrum as in milk which was confirmed by Richards (1930) although in these determinations the difference was less marked. Herford (1871) found it in normal urine and Schiaparelli and Peroni (1881) in horse

urine. Bradley (1907) determined the amount of manganese in two clams, Unio and Anodonta, from Madison Lake and found that about 1 per cent of the dried tissue was manganese; it was also present in the eggs of these molluscs to the extent of 0.6 to 0.8 per cent. Later (1910) he found it always present in Modiola modiolus (of the Massachusetts region) even when the animals had been starved for 3 weeks. But in no

TABLE 1

Distribution of manganese in different tissues

(Milligrams per 100 grams wet tissue)

SPECIES	LUNG	LIVER	KIDNEY	AUTHOR
Rabbit		0.237–0.351	0.087–0.101	Bertrand and Medigreceanu, 1913
Rabbit (8)	0.036	0.178	0.147	Lund, Shaw and Drinker, 1921
Cat (15)	0.031	0.341	0.136	Lund, Shaw and Drinker, 1921
Dog	0.010	0.306	0.106	Bertrand and Medigreceanu, 1913
Dog (8)	0.023	0.238	0.087	Lund, Shaw and Drinker, 1921
Guinea pig (2)	0.00		0.000	Lund, Shaw and Drinker, 1921
Rat (2)	0.00		0.110	Lund, Shaw and Drinker, 1921
Macacus rhesus	0.055	0.120	0.155	Mella, 1923
Ox	0.010	0.298	0.084	Bertrand and Medigreceanu, 1913
Calf	0.011	0.290	0.063	Bertrand and Medigreceanu, 1913
Pig	0.023	0.265–0.317	0.109–0.128	Bertrand and Medigreceanu, 1913
Horse	0.006	0.280	0.077	Bertrand and Medigreceanu, 1913
Chicken	0.010	0.416	0.217	Bertrand and Medigreceanu, 1913
Chicken (2)	0.00		0.124	Lund, Shaw and Drinker, 1921
Canary	0.010	0.380	0.238	Bertrand and Medigreceanu, 1913
Turtle (2)	0.00			Lund, Shaw and Drinker, 1921
Man	0.20	0.170	0.061	Reiman and Minot, 1920
Maximal variation	0.00–0.055	0.120–0.351	0.061–0.155	
Median mammals	0.016	0.273	0.104	
Median birds	0.010	0.398	0.217	

instance were the quantities as large as observed with Uniodae. All tissues gave a positive manganese reaction but it was most marked in the nephridial organs. In Pecten the results were less uniform, in some specimens the element was present in rather large quantities, in others only in traces, the content presumably varying with the surroundings. In Mytilus edulis manganese was present in all specimens tested, but only in traces. Bertrand and Medigreceanu (1913) who give the older

literature on the distribution of manganese in the tissue found that there is no considerable variation of the manganese content in the same species. The distribution is within the same range with most mammals, but liver and kidney of birds appear to show distinctly higher values (table 1). In eggs only the yolk appears to contain manganese and the eggwhite was found to be free of this element. Already Pollacci (1870) had detected manganese in chicken eggs and later McHargue (1924); and Richards (1930) found that their manganese content increases during maturation in the ovaries. Reiman and Minot (1920) determined the distribution of manganese in different tissues of man, Lund, Shaw and Drinker (1921) in a number of species and Mella (1923) in the organs of Macacus rhesus. The data for lung, liver and kidney are collected in table 1.

This table indicates that the liver contains normally by far the largest amount of manganese (0.273 mgm. per 100 gm. wet tissue), next ranks the kidney (0.104 mgm.); whereas the spleen contains a median of 0.054 mgm. (Reiman and Minot, 1920; Lund, Shaw and Drinker, 1921; Mella, 1923). From other glandular structures the adrenals of man contained 0.013 mgm., the pancreas 0.076 mgm. and the lymph nodes 0.021 mgm. (Reiman and Minot, 1920). The values for the brain as determined by Lund, Shaw and Drinker (1921) for rabbits, cats and dogs, by Mella (1923) for Macacus rhesus and by Reiman and Minot for man give a median of 0.035 mgm.; according to Lund, Shaw and Drinker the spinal cord of cats contained 0.059 mgm., being, therefore, of the same order as that of the brain. The manganese content of the intestinal tract shows considerable variations in different sections. Lund, Shaw and Drinker (1921) found as median value for the stomach of rabbits, cats and dogs and Reiman and Minot (1920) for that of man 0.036 mgm., for the small intestine it is 0.037 mgm. and for the colon it is 0.083 mgm. From other structures the bone contained 0.101 mgm. (Lund, Shaw and Drinker, 1921), the muscle tissue 0.024 mgm. (Reiman and Minot, 1920; Lund, Shaw and Drinker, 1921), the heart 0.046 mgm. and the lungs 0.016 mgm. Bertrand and Medigreceanu (1913) determined the manganese content of eggs of chicken and canaries as 0.063 and 0.054 mgm. *It appears, therefore, that like other metals manganese is mainly stored in the liver, that the kidney ranks next and that it is partly excreted into the colon.*

Interesting are also the high values for the liver and kidneys of birds which are nearly twice as great as those found for mammals. This variation of the manganese content of different species becomes still

more prominent in table 2 which gives some data taken from a paper of Bertrand and Medigreceanu (1913).

This table shows to which extent the surroundings may affect the manganese content of the animals as indicated by the great variations with some of the fishes, crustacea and molluscs.

THE ABSORPTION OF MANGANESE FROM THE INTESTINAL TRACT. The question whether and to what extent manganese is absorbed from the

TABLE 2

Manganese content of different animal species

(Total amount of managnese in milligrams per 100 grams fresh tissue)

(Bertrand and Medigreceanu, 1913)

A. Mammals:		F. Crustacea:	
White rat (intestine emptied)	0.030	Crawfish	9.144–11.11
White mouse (intestine emptied)	0.036		
White mouse (intestine filled)	0.190	Shrimp	0.147
B. Birds:		Woodlouse	0.700
Turtle dove (intestine filled)	0.207	Barnacle	0.180
C. Amphibia:		G. Molluscs:	
Horned viper (intestine empty)	0.180	Snail (edible)	
Red frog (intestine empty)	0.390	without shell	2.843
Green frog (intestine filled)	0.627	shell alone	4.236
Salamander	1.017	Lymnea	0.477–4.145
D. Fishes:			
Herring	0.121–0.900	Oyster	0.100
			0.649
Sardine	0.116	Oyster shell	0.595–0.818
Plaice (flounder)	0.238		
Mackerel	0.056	H. Annelides:	
Whiting	0.094	Leech	<0.040
Shark	0.611	I. Echinoderms:	
E. Insects:		Starfish	0.094
Cabbage butterfly	0.248	Sea cucumber	0.040
Gyrinus	0.702		
Locust	0.381		

intestinal tract is important in regard to the hazards to which workers handling manganese ores are exposed. Orfila (1826), Gmelin (1837) and Huenefeld and Wibmer (1831) (quoted from Harnack) had found that introduction of simple manganese compounds into the stomach causes toxic symptoms only when very large doses are given which produce corrosion of the gastric walls and of the intestine. Kobert (1883) fed manganese citrate to rabbits and observed very little absorption from the intestinal tract. A better absorption was noted with larger doses

which caused gastritis. Cahn (1884) also stated that only very little manganese is absorbed from the intestinal tract. Reiman and Minot (1921) determined the solubility of franklinite, rhodonite and manganese dioxide in gastric juice, they found that all are soluble to a certain extent depending upon the acidity and duration of the contact and that this was sufficient to secure absorption of manganese into the bloodstream. Two persons drank a suspension of 8 grams of franklinite in water alone and followed by the intake of 50 grams of cooked meat. Blood samples were taken at intervals of 1, 3, 6, and 24 hours after the administration. There was no difference in both series; the manganese level in the blood increased rapidly during the first hours and then rapidly returned to normal as indicated by the following data:

Time after administration.....	0	1	3	6	24 hours
Manganese in mgm. per 100 gm.....	0.0105	0.014	0.012	0.0105	0.0105

Similar results were reported by Drinker and Shaw (1921) after intravenous injections of manganese dioxide suspensions into cats.

The reason for this rapid disappearance from the circulation with oral administration is that the solubility in gastric juice is rather slow and that on the other hand the rate of elimination is too rapid to allow an accumulation in the blood. Von Oettingen and Sollmann (1927) observed no toxic effects in birds (pigeons and roosters) after administration of metallic manganese as indicated by the red cell count and the weight curve.

It appears, therefore, that the absorption of manganese from the gastrointestinal tract is very slow and incomplete under normal conditions and that there is only a temporary increase of the manganese level in the blood.

THE EXCRETION OF MANGANESE. Wichert (1860) (quoted from Harnack) detected manganese in the bile of cats after its oral administration. After the oral administration of manganese citrate to rabbits Kobert (1883) found most of it excreted with the feces and only traces in the urine. Only when large doses were given which developed gastritis was the urinary excretion more marked. Also Cahn (1884) stated that manganese is mostly excreted with the feces. Harnack and Schreiber (1901) with the oral administration of manganese peptonate and manganese phosphate to rabbits and Bargero (1906) who administered manganese orally to dogs confirmed the results of Kobert. After the administration of manganese peptonate to patients with biliary fistula,

manganese could be detected in the bile. According to Bargero (1906) the biliary excretion increases with the manganese content of the liver, but Benedetti (1906) who injected manganese citrate subcutaneously into dogs stated that larger doses tend to decrease the biliary excretion. Reiman and Minot (1921) reported on one person who drank 25 grams of franklinite suspended in water in the course of three days. During this time and several days afterwards 24 hour samples of urine and of feces were collected, and 500 cc. of the urine and 2 grams of the feces were analysed for manganese and the total amount of the excreted material was calculated. They found that it is mainly excreted through the intestine and in no instance could more than a trace be detected in the urine. Administration of franklinite to patients with biliary fistulas showed that the manganese content of the bile which is normally very low, ranging from 0.003 to 0.010 mgm. per 100 grams, was materially increased after the administration of the ore. After the administration of 5 grams in three doses to two patients the manganese content of the bile went up to 0.111 mgm. and 0.120 mgm. per 100 grams on the following day, whereas another patient after the same dose showed only a moderate increase. All three persons showed a slow return to the normal level. Handowsky, Schulz and Staemmler (1925) found in rabbits with subcutaneous injection of manganese chloride, manganese citrate and a manganese pyrocatechol derivative that about 50 per cent were excreted with the feces but only traces with the urine. They found that bile, ascitic fluid and pus contained comparatively large quantities. Orent and McCollum (1931) stated that manganese passes through the placenta.

From these data it appears that after oral and subcutaneous administration manganese is excreted mainly by the feces, partly in the bile, and presumably also through the colon as indicated by the large manganese content of this organ. The urinary excretion is, however, very moderate as may be expected from the rapid disappearance of the metal from the circulation.

DISTRIBUTION OF MANGANESE AFTER ITS ADMINISTRATION. Kobert (1883) stated that in rabbits after the subcutaneous injection of manganese citrate all organs, especially the liver, kidney and the intestine contained manganese. After intravenous injection of the same compound into rabbits Cahn (1884) found most in the intestinal walls and large quantities in the liver and kidneys. Harnack and Schreiber (1901) fed manganese peptonate to rabbits in doses of 2 grams daily for from three to four weeks and detected large quantities in the spleen, liver, stomach, and colon, but less in the kidneys. Benedetti (1906)

injected manganese citrate subcutaneously into dogs and found the metal in the liver and kidneys as early as twelve hours after the injection. Similar experiments were reported by Bargero (1906) with oral administration of manganese to dogs. Handowsky, Schulz and Staemmler (1925) stated that after the subcutaneous injection of manganese chloride, manganese citrate and of a manganese pyrocatechol derivative large quantities were deposited in the bones, spleen and brain; the content of the liver being variable and the lungs containing comparatively small quantities. Reiman and Minot (1921) determined the manganese content of the organs of dogs which had been fed franklinite, pyrolusite and rhodonite over a long period. Their results and those reported by Mella (1923) with intraperitoneal injections of manganese chloride into monkeys and of Handowsky and his collaborators with subcutaneous injections into rabbits are given in table 3.

Reiman and Minot's experiments show that with oral administration the manganese content of brain and spinal cord is hardly increased as compared with the normal, and that the concentration in the hemispheres, the spinal ganglia and the cord is of the same order. The same holds true for the lungs, spleen and muscle indicating that the manganese in circulation is not filtered off by these organs like colloids. The stomach, the small intestine and the colon show considerable increase which is least with the stomach, more marked with the small intestine and most pronounced with the colon, presumably on account of the excretion of the metal into the latter. *From these data it appears that liver and kidney are the main storage places of the metal.* The storage in the bones as stated by Handowsky and his co-workers is very likely, but a quantitative comparison is impossible, because no comparative data are available. The experiments of Richards (1930) who gave from 0.054 to 0.47 gram of manganese daily with the food to pigs showed, however, no evidence of a very marked accumulation in the liver, which may perhaps be explained by differences in the solubility of the manganese in the gastric juice.

The figures presented by Mella with intraperitoneal injection of manganous chloride show a tremendous increase of the manganese content of both liver and brain as compared with the normal values, but the increase of the liver values is also by far the greater.

The results obtained by Handowsky, Schulz and Staemmler with subcutaneous injection of the manganese pyrocatechol derivative and manganous chloride in rabbits indicate that with this method of administration the distribution of manganese in different organs is quite different

TABLE 3

Distribution of managenese in organs after its administration

(Milligrams per 100 gram wet tissue)

ORGANS	DOGS, ORAL ADMINISTRATION REIMAN AND MINOT, 1920		MACACUS RHESUS, INTRAPERITONEAL INJECTION MELLA, 1923		RABBITS SUBCUTANEOUS INJECTION HANDOWSKY, SCHULZ AND STAEMMLER, 1925		
	Ores*	Normal†	$MnCl_2$	Normal	$MnCl_2$	Mnpyrocatechol	Normal†
Brain (hemispheres)...	*0.030* 0.023–0.042	0.044	0.122–0.173	0.032	1.154	1.514, 2.538	0.062
Spinal cord.....	*0.045* 0.024–0.088	0.044					
Lung.....	*0.044* 0.024–0.056	0.023			0.287, 1.335	0.124, 0.472	0.036
Liver.....	*0.600* 0.276–0.900	0.238	4.906–8.88	0.120	0.08, 2.642	0.045, 3.582	0.238
Spleen.....	*0.037* 0.025–0.112	0.022			3.333, 5.260	12.5, 0.736	0.054
Kidney.....	*0.151* 0.110–0.216	0.087			0.431, 3.720	0.963, 1.372	0.147
Stomach.....	*0.098* 0.059–0.211	0.043					
Duodenum.....	*0.131* 0.051–0.377						
Small intestine.....	*0.190* 0.062–0.420	0.028					
Colon.....	*0.308* 0.060–0.396	0.080					
Pancreas.....	*0.154* 0.089–0.284						
Heart.....	*0.054* 0.024–0.300				1.076, 0.50	0.362, 0.189	
Bone (marrow).....	*0.135* 0.043–0.550	0.101			6.817		0.101
Muscle.....	*0.024* 0.016–0.042	0.020			0.647, 1.661	0.0, 0.0	0.022
Basal ganglia.....	*0.049* 0.042–0.138						
Omentum.....	*0.013* 0.006–0.130						
Bile.....	*1.60* 0.666–3.68						

* These animals received a total of from 228 to 768 grams of franklinite, rhodonite or manganese dioxide in the course of from 270 to 477 days.

† The normal values are from the paper of Lund, Shaw and Drinker (1921). The figures below the medians indicate the extremes.

from that with oral administration and very irregular especially in regard to the concentration in the liver. Special attention is drawn to the fact that only manganese injected as chloride could be recovered from the muscle, but not if injected as the pyrocatechol derivative.

With the intravenous administration of suspensions of manganese dioxide, as reported by Drinker and Shaw (1921), the distribution is somewhat different. Although the available data are less complete the increase of the concentration in the intestinal tract is considerably less marked than with oral administration. Unfortunately there are no data for the manganese content of lungs and spleen which might indicate a filtration in the hematopoietic system. These experiments do not reveal much retention of manganese in the central nervous system.

TABLE 4

Per cent of injected manganese recovered one hour after the intravenous injection of a suspension of manganese dioxide

(Drinker and Shaw, 1921)

	LIVER	LUNGS	SPLEEN	BLOOD	OTHER ORGANS
20 cats	38.3	47.0	4.7	0.0	10.0
3 rabbits	81.0–85.6	0.2 – 1.2	0.55–2.5	0.0–0.7	13.3–16.5
3 dogs	64.0–83.0	0.52–14.0	2.0 –4.0	0.9–3.9	9.1–16.1
2 guinea pigs	76.0–85.0	0.8 – 2.1	1.4 –1.7	0.3–1.2	12.5–19.0
2 rats	94.0–98.0	0.5 – 0.6	0.8 –1.9	0.0	2.0– 3.6
2 chickens	84.0	0.0 – 0.4	1.7	0.0	13.9–14.3
2 turtles	94.0–88.0	3.0 – 7.5		0.0	3.0– 4.5

Drinker and Shaw (1921) reported that after the intravenous injection of suspensions of manganese dioxide to cats the liver, lungs and spleen contained 90 per cent of the injected material and with other animal species the distribution appears not to be materially different as indicated in table 4.

As already mentioned, the intravenous injection of a suspension must necessarily give a different distribution and for this reason these experiments do not permit conclusions as to the distribution of manganese in the organism after the ingestion of manganese compounds. The same holds true for the results reported with subcutaneous and intraperitoneal administration.

PHARMACOLOGY OF MANGANESE. Gmelin (1824) (quoted from Harnack) found that the oral administration of manganous sulfate in large doses to dogs causes vomiting, gastritis and paralysis, and the intrave-

nous injections produce shocklike symptoms, hyperemia of the organs and also an increase of the excretion of bile. Kobert (1883) also found that in dogs the repeated subcutaneous injection of small doses of manganese citrate causes nausea, vomiting and icterus; most of his animals developed parenchymatous nephritis which passed into partial or complete cirrhosis. According to Kobert, Hoppe (1858) had claimed that manganese sulfate in certain concentrations stimulates the isolated heart and the isolated intestine, and that it causes vasoconstriction and later paralysis. Harnack (1875) reported that manganese sulfate causes a motor depression in frogs, reduction of the sensitivity and of reflex excitability. He found that the muscles were not affected, because they responded promptly to direct and indirect stimulation. With doses up to 10 mgm. the animals recovered from the depression within one to two hours, larger doses of from 10 to 25 mgm. were fatal. Kobert (1884) using manganese citrate confirmed these results and noted also depression of the respiration and of the heart, in which first the motor ganglia and later also the muscle were affected. With intravenous injections into dogs and rabbits he noted a fall of the blood pressure, with a subsequent rise during the convulsive stage. The convulsions were followed by complete depression and the animals died of paralysis of the respiration while the heart continued to beat for some time. This was confirmed by Cervinka (1929). Kobert also noted no effect on the striated muscle. There seems to be a characteristic difference between frogs and mammals inasmuch as in the latter the heart survives the respiratory depression. This was also demonstrated by Cahn (1884) who found that in rabbits death may be postponed with artificial respiration. On the other hand Cervinka (1929) claimed there is a fundamental difference between rabbits and dogs as with intravenous administration the former first show paralysis of the vasomotor mechanism, which caused the fall of the blood pressure and slowing of the pulse and that in this case the asphyxia was secondary. Whereas with dogs the fall of blood pressure is due to cardiac depression. Sato (1929) claimed a difference in the toxicity of bivalent and trivalent manganese. In rabbits the intravenous injection of 0.3 mgm. per kilo of manganous salts was already distinctly toxic, whereas the corresponding manganic compound caused no toxic symptoms in doses as high as 7 mgm. per kilo and even 15 to 20 mgm. were not fatal. A similar difference of the toxicity was also noted in regard to the fall of the blood pressure and it appears that the bivalent manganese is about ten times as toxic as the trivalent. Sato also stated that small doses of manganese stimulate the gas exchange, whereas large doses cause a depression presumably on

account of the fall of blood pressure and the reduced tissue respiration. Handowsky, Schulz and Staemmler (1925) pointed out a fundamental difference between the acute and the chronic manganese poisoning. The acute poisoning is only caused when suddenly a high concentration of manganese is produced in the blood; it is characterized by an ascending paralysis of the central nervous system. Such symptoms were also observed in rabbits by Findlay (1924) with the subcutaneous injection of from 10 to 16 mgm. of manganous chloride. With lower concentrations in the blood, nervous symptoms are not seen, but the liver shows degeneration. The velocity of these changes depends on the amount of metal absorbed within a certain period. With chronic poisoning the Kupffer cells of the liver are first affected. Handowsky observed his animals only for a few weeks, Mella (1923) injected intraperitoneally small doses of manganese chloride into monkeys (Macacus rhesus) over a long period (up to 18 months). These animals developed movements of the choreic and the choreoathetoid type, later they showed a rigidity of the muscles and disturbances of the motility. In the later stage they developed fine tremors of the hands and finally a peculiar contracture of the hands with the terminal phalages extended.

THE EFFECT OF MANGANESE ON THE BLOOD. As has been stated, Burin de Buisson as early as 1852 had claimed that manganese is an essential constituent of the red blood cells and that its administration is an effective treatment of anemias. Schwarz and Pagels (1923), feeding manganese dioxide and pyrolusite ore to cats, found that after four weeks the animals developed an increase of the hemoglobin and of the red blood cells, followed after further administration for another month by a decrease of both. They noted that the pure preparation was more effective than the ore, presumably on account of the greater solubility in the gastric juice. There was also a considerable variation in the sensitivity of different animals. Titus, Cave and Hughes (1928) working with young rabbits made anemic by an exclusive milk diet, found that daily doses of 0.5 mgm. manganese had a definite favorable effect when added to the milk which contained also 0.5 mgm. of iron. This was, however, not confirmed by Waddell, Steenbock and Hart (1929) and also by Mitchell and Miller (1931) who fed 0.1, 0.05, 0.025, and 0.01 mgm. manganese daily and observed only a negligible supplementary action to iron in the synthesis of hemoglobin. They stated, however, that it may have a slight stimulative effect on growth and food intake. Orent and McCollum (1931) also assume that manganese plays no rôle in the regeneration of blood. Myers and Beard, on the other hand, believed that manganese (lowest optimal dose 0.1 mgm. daily)

when added to the iron-milk diet speeds up the regeneration of blood in anemic young rats. They assumed that iron is the essential element in the process and that manganese has some catalytic action. Beard, Rafferty and Myers (1931) found that addition of manganese to the iron-milk diet is no more effective in preventing nutritional anemia, than is iron alone. The same holds true for development of hemoglobinemia (Myers, Beard and Barnes, 1931). Although addition of manganese to the iron-milk diet does not increase the reticulocytosis in young anemic rats, it causes a marked drop of the reticulocytes on the 5th or 6th day with a more rapid increase of cells and hemoglobin than observed with iron alone. This may suggest that in these doses manganese speeds up the maturation of red blood cells (Beard, Baker, and Myers, 1931).

EFFECT OF MANGANESE ON THE GROWTH. It has been demonstrated before that manganese is essential for the growth of plants. Levine and Sohm (1924) demonstrated that it has also a favorable effect on the growth of young rats. They found that manganous sulfate in concentrations of 1:2000, 1:4000 and 1:10000 given with the food had a marked catalytic action on the growth, and the manganese-fed animals were more active than the controls and the hair was sleeker, longer and thicker. They claimed that the offspring of such animals grew somewhat faster than the average young rat. McCarrison (1926) reported that feeding of manganous chloride to young rats to the extent of 0.037 mgm. daily had a markedly favorable effect on their growth. Larger doses, on the other hand, such as 0.889 mgm. had a progressively increasing retarding effect which became manifest after 37 days of feeding apparently due to accumulation. Later experiments of the same author (1929) seem to indicate that manganese in foodstuffs may act differently than manganese salts. McHargue (1926) assumed that manganese compounds as they occur in green leaves and seeds of mature plants and in the vital organs of animals have much more active biological potency than could be expected from feeding equal proportions of a crystalline salt of manganese in a synthetic diet. Bertrand and Nakamura (1928) confirmed the findings that manganese plays some important rôle in the animal metabolism. Also Kemmerer, Elvehjem and Hart (1931) found that addition of 0.01 mgm. of manganese as manganese chloride per mouse daily to the diet of whole cows' milk, supplemented with iron and copper, has a favorable effect upon growth of mice. On the other hand Orent and McCollum (1931) found that young rats fed with a manganese free diet grow to maturity in an apparently normal manner, but that the offspring of such animals are undersized and inferior in appearance and that they are not fed by

their mothers. Male rats on a manganese free diet also developed normally but showed degeneration of the testes. Addition of from 0.005 to 0.05 per cent manganese in form of manganese chloride to the diet prevented this degeneration. They assumed that manganese is concerned with the production of a hormone by the anterior lobe of the hypophysis which is essential for the functioning of the testes and for the proper development and functioning of the mammary tissue.

AUTOPSY FINDINGS IN ANIMALS TREATED WITH MANGANESE. Kobert (1883) in some of his animals which had received subcutaneous injections of manganese citrate noted nephritis and cirrhosis of the kidneys. After intravenous injections into dogs Bargero (1906) observed fatty degeneration of the liver. Findlay (1924) injected subcutaneously into rabbits 10 to 16 mgm. of manganous chloride and the animals died within 22 to 48 hours in a semi-comatose state. The urine contained considerable quantities of albumen, red blood cells, casts and traces of bile pigments. The liver was slightly enlarged, friable and of a pale-yellow color often with reddish mottlings. It showed fatty degeneration especially at the periphery of the lobules; a few Kupffer cells were swollen and prominent, but there was no proliferation of the endothelial cells. All organs showed more or less marked congestion, the liver contained small hemorrhagic areas; the glomeruli were also congested and frequently blood was found in the intercapsular space and the kidneys gave evidence occasionally of fatty degeneration. In another series of experiments Findlay injected every second day from 3 to 5 mgm. of manganous chloride and these animals lived from five to nine weeks. At autopsy they showed a biliary type of cirrhosis of the liver, but no fatty degeneration. Similar changes were also observed in rats after the oral administration of 0.3 to 0.4 gram of manganese chloride daily. Handowsky, Schulz and Staemmler (1925), whose animals survived the subcutaneous injections of different manganese compounds for three to four weeks, noted fatty degeneration of the liver beginning in the Kupffer cells and extending to other cell structures; occasionally there were also degenerative changes in the kidneys and in the heart. Mitotic processes observed in the liver cells were interpreted as symptoms of regeneration. They also saw destruction of the erythrocytes, indicated by the formation of hemosiderin. The difference between these findings and those reported by Findlay may be explained by the more rapid course of the poisoning in Hadowsky's series. Mella (1923) who injected monkeys intraperitoneally with small doses of manganous chloride over a period of 18 months found acute hepatitis and areas of liver necrosis. In the nervous system he noted the following changes: The

cortex of the brain showed slight but evenly distributed chromolysis, with vacuolated nerve cells but no gliosis or myelin degeneration. The neurons of the cortex contained fat droplets. In the putamen and caudate nucleus the cells were shrunken and pyknotic, many having lost their nuclei. There were scattered areas of gliosis which occasionally were quite dense. Many of the fibers connecting the caudate nucleus with the putamen had lost their myelin sheath. The globus pallidus showed degenerative changes. In one animal a definite loss of the myelin sheath in the ansa lenticularis was noted. The cerebellum gave essentially a negative picture, the pons as a whole was normal and also the spinal cord. The peripheral nerves exhibited no pathologic changes. Gruenstein and Popowa (1929) fed daily doses of from 2 to 3 grams manganese dioxide to rabbits and after the death of these animals noted degenerative changes in liver, spleen, heart, kidneys and adrenals. All animals showed severe changes of the central nervous system, characterized by a diffuse degeneration of the nerve cells in the corpus striatum and of the small cells of the caudate nucleus and of the pentamen. They also saw degenerative changes of the endothelium of the plexus choreideus and inflammation of the pia. Matsumura (1933) studied the effect of subcutaneous injections of manganese chloride in rabbits and dogs. In rabbits the degenerative changes in the liver were more marked than in dogs. The former species showed hyperemia, hemorrhages, dilatation of the veins and regressive changes in the liver cells of different degree. In severe cases there were also sharply defined degenerative processes in the periphery of the acini which occasionally showed formation of connective tissue. This could also be observed in Glisson's sheath giving the picture of beginning cirrhosis of the liver. In dogs the degenerative changes in the liver were less marked and consisted mainly in stasis, small hemorrhages, and slight regressive changes of the liver cells. On the other hand he found that pathologic changes of the central nervous system were comparatively slight in the rabbit. They consisted of stasis and hemorrhages in the brain and shrinkage of the ganglionic cells in the superficial layers of the cortex of the hemispheres. Similar changes were found in the entire brain and were not especially marked in the interbrain and the corpus striatum. Neither rabbits nor dogs showed the extrapyramidal symptoms observed in man. The central nervous system of the dog exhibited, however, various changes of the ganglionic cells, mainly with edematous changes, weblike shrinkage and swelling, total chromatolysis and vacuole-formation in the ganglionic cells. There was swelling and proliferation of the glia cells and glia nodes were observed. The cells

of the vascular walls showed swelling and proliferation. The ganglionic changes were observed in the superficial layers of the hemispheres as in the rabbits and also in the thalamus and corpus striatum. Glia changes were found in the striatum, thalamus and in the floor of the fourth ventricle. Changes of the vascular walls were most frequent in the thalamus and the striatum.

Although Matsumura does not assume a specific relation between the pathological changes in the liver and the central nervous system, other experiments, especially those with oral administration appear to indicate that the degenerative changes in the liver are the earlier symptoms of manganese poisoning and that pathologic changes of the central nervous system occur at a later stage.

TABLE 5

*Reports on industrial manganese poisoning**

NUMBER	AUTHOR	YEAR	NUMBER OF CASES
1	Couper	1837	5
2	von Jaksch	1901	3
3	Embden	1901	4
4	Seiffer	1904	1
5	von Jaksch	1907	1
6	Casamajor	1909	9
7	Seelert	1913	1
8	Edsal, Wilbur and Drinker	1919	3
9	Davis and Huey	1922	2
10	Charles	1922	3
11	Gayle	1925	6
12	Charles	1927	7
13	Hilpert	1929	1
14	Meyer	1930	1
15	Hiplert	1930	13
16	Schwarz	1931/32	2
			62

* Since this paper went to press, 7 more cases of manganese poisoning have been reported. Baader (1932, 1934) published 1 case of a man engaged in unloading manganese ores, and 2 cases of workmen engaged in a dry cell plant. One case was reported by Ashizawa (1927), and 4 cases by Owen and Cowen (1934), so that there are altogether 70 cases of manganese poisoning on record.

INDUSTRIAL MANGANESE POISONING. The first report on this subject is that of Couper (1837) on five cases of manganese poisoning observed in workers handling powdered manganese dioxide. In three of these the cause of the disease was recognized very early and the patients

recovered after the discontinuation of their occupation. Altogether there are 70 cases of manganese poisoning reported in the literature which are collected in table 5.

Most of these workmen were exposed to the dust of pyrolysite or of manganese ores in mills or in discharging boats. The absorption takes place presumably through the gastro-intestinal tract although it is possible that some is inhaled through the lungs. All observers report a peculiar masklike expression of the face, involuntary laughing and less frequently crying (2, 4, 8, 11), the voice is low and the speech is blurred and scanned. Most patients show rather early a peculiar stiffness of the muscles which results in a spastic gait and in propulsion and retropulsion (jerking forward or backward while walking or stopping). Occasionally also a peculiar gait on the metatarsophalangeal joint is observed (Charles, 1927). Intension tremor especially of the hands is practically always present; micrography is frequently observed (3, 4, 8, 11, 15). The tendon reflexes are often increased (2, 3, 4, 5, 7, 8, 10, 12, 15). There are usually no sensory disturbances aside from paresthesias which have been observed occasionally in the beginning of the disease (3, 6, 11, 16). Psychic changes are not a rule but a certain mental dullness has been observed occasionally (2, 8, 14, 16). Gayle (1925) reported in two out of six cases a pronounced lack of sociability. Insomnia was only reported once (Casamajor, 1909). In a few cases salivation and lacrimation have been noted (4, 10, 12). Visual and acoustic disturbances are not frequent, the latter have only been reported by Casamajor (1909). The function of the intestinal tract and of the bladder is not affected. The urine contains manganese only in fresh cases and is usually without pathologic constituents; von Jaksch (1901) reported a temporary glucosuria in one case. The blood picture if reported is practically always normal, only Schwarz (1932) found in his case 6.28 million erythrocytes, 76 per cent hemoglobin and 40 per cent lymphocytes. The spinal fluid as far as studied never showed pathologic changes.

Although most of the patients show extrapyramidal symptoms the reports of von Jaksch (1907) and Hilpert (1930) indicate that they may develop signs of pyramidal lesions in the latter stage of the disease. The clinical symptoms observed in manganese poisoning have been compared with multiple sclerosis (Embden, 1901), and with epidemic encephalitis (Cohn, 1928). It may be distinguished from the latter by the absence of pathologic changes in the background of eyes (Meyer, 1930). Hilpert (1930) mentions some similarity with metencephalitis, paralysis agitans and chronic carbon monoxide poisoning. The simi-

larity to the latter is illustrated by the report of Schlockow (1879). Stoecker (1913) was the first to direct the attention to the similarity of manganese poisoning with progressive lenticular degeneration (Wilson's disease) which is associated with a peculiar degeneration of the liver. A relation between liver changes and nervous symptoms was also made by Charles (1927) who reported that some of his patients showed symptoms of hepatic insufficiency. In this connection may be mentioned the observation of a temporary alimentary glucosuria in one of the cases of von Jaksch. The animal experiments reported above with acute manganese poisoning as well as with chronic manganese poisoning over long periods showed degenerative changes of the liver. Some of the cases reported in the literature had a history of alcoholism, with most of them this point is not stressed but it appears very likely that workmen of a rather low type as employed in manganese mills and in discharging of boats are accustomed to the use of strong alcohols and in this way a predisposition may be produced for the toxic effects of manganese on the liver. The only autopsy of a case of manganese poisoning in the literature is that of Casamajor (1916) which showed lesions of the medulla and biliary cirrhosis.[1] On the other hand it is quite likely as pointed out by Gayle (1925) that the individual susceptibility may play an important rôle in the development of manganese poisoning.

The course of manganese poisoning is progressive and there seems to be no effective curative treatment. Charles (1927) reported, however, that he saw distinct improvement in the conditions of some of his patients with liver therapy, although in no instance was a cure obtained. It is therefore important that manganese poisoning be recognized at an early stage. Schwarz and Pagels (1923) suggested the periodic control of the erythrocytes of the blood and of the hemoglobin as adequate and considered a rise of the red cell count as the first symptom of manganese toxicosis. But in none of the cases reported in the literature with the exception of that of Schwarz (1932) was a pathologic change of the blood picture reported, and therefore it appears that such control may not be very efficient. On the other hand a periodic determination of the hepatic function may lead to early detection, and abuse of strong alcoholic drinks should be avoided. In view of the lack of therapy the prophylaxis is the most important measure. That this may be quite effective in reducing the incidence of manganese poisoning is shown by the report of Wilbur (1932) that improved dust-collecting

[1] Further reports on autopsies were published by Ashizawa (1927) and by Canavan, Cobb and Drinker (1934).

systems reduce the occurrence of manganese poisoning in ore mills and in similar places.

Von Jaksch (1901) had assumed that bivalent manganese is more toxic than trivalent and, therefore, more liable to produce manganese poisoning, which was confirmed by Sato (1929); but from the history of the cases reported it appears to be only of minor importance in the production of industrial manganese poisoning.

TOXICITY OF MANGANESE. The toxicity of manganese varies for different salts and for the bivalent and trivalent manganese ions.

For *manganous chloride* Reichert (1881) gave the fatal concentration for fishes as 0.3 gram manganese per liter. With subcutaneous injections Handowsky, Schulz and Staemmler (1925) gave the fatal dose as 50 mgm. per kilo for mice, guinea pigs and rabbits. With intravenous injections Cervinka (1929) found that the minimal fatal dose was 56 mgm. per kilo in dogs and 18 mgm. per kilo in rabbits.

Manganous citrate injected subcutaneously is, according to Handowsky and his co-workers (1925), more rapidly fatal than manganous chloride. In the same dose of 50 mgm. per kilo there is apparently more rapid absorption from the site of the injection. Kobert (1883) found that the subcutaneous injection of 6 to 8 mgm. (MnO) kills dogs in two days and doses of 13 to 14 mgm. per kilo in 24 hours. The same doses were fatal for cats; rabbits are killed with doses of 12 to 13 mgm. per kilo and guinea pigs with 28 to 30 mgm. per kilo. Rabbits and guinea pigs show no symptoms during the first hours, later thay develop convulsions ending in paralysis, or with smaller but fatal doses they die from progressive depression.

Potassium permanganate is used on account of its oxidizing action as an antiseptic and antidote in alkaloid poisoning. In this compound manganese exists as an anion and one has, therefore, to consider the toxicity of both the potassium and the manganese radicle. The subcutaneous injection of aqueous solutions causes local necrosis of the skin (Harnack and Schreiber, 1901). According to Levine (1929) with oral administration 10 grams are fatal to the horse, 0.24 gram per kilo to rabbits; for man 5 grams may be fatal but larger doses have been tolerated. Pills containing 0.12 gram, given for therapeutic purposes, produced pain of the upper digestive tract, cardiac disturbances, collapse and cyanosis. Several such doses may give rise to abortion. According to De Jongh (1933) this is due to a direct effect on the uterine muscle on account of the oxidizing action of the permanganate and is not caused by the manganese ion. In man poisoning is characterized by vomiting, inability to speak (without loss of consciousness), lividity of lips, chin

and fingers, and swelling and blackening of the tongue. Marked edema of the glottis has been observed and also ulceration and corrosion of the upper digestive tract. Kidney disturbances may occur. In some instances the respiration stopped after the cardiac arrest. At autopsy the following findings were reported: corrosions of the mouth and of the esophagus (not of the stomach), pulmonary edema, broncho-pneumonia, and occasionally degenerative changes of the heart, liver and kidneys.

THERAPEUTIC USE OF MANGANESE COMPOUNDS. Manganese has been used in a great variety of conditions, but so far it has not been generally accepted in any field, except in the form of potassium permanganate as an antiseptic and antidote in alkaloid poisoning. De Buscher (1904) found it to be of limited value in the treatment of morphine poisoning. Widenmann (1915) demonstrated its usefulness in the treatment of snake bites. Recently Taylor recommended manganese sulfate in concentrations of 1:1000 for the treatment of fungus infections of the foot.

Kugler (1831) suggested the use of manganese in scrophulosis. Hannon (1849), Pètrequin (1852), and Burin de Buisson (1852) advocated its administration in chlorosis and in anemic conditions and recently Faludi (1930) reported good results with the administration of a manganese albumen mixture in 17 cases of secondary and 6 cases of pernicious anemia. The former exhibited rapid improvement and five of the patients with pernicious anemia showed complete remission. Camescassé (1923) gave doses of 0.07 gram of manganese dioxide in tablets daily for two weeks to debilitated and undernourished children and claimed a greater speed of recovery, a greater and more rapid gain in weight and apparently also of the growth as compared with untreated children of the same type. Nott (1925) advocated the rectal administration of potassium permanganate (1–1½ grain per pint) combined with the oral administration of thyroid in a great number of pathologic conditions and from the comments on this paper it appears that it is used in this way as a roborant also by other practitioners in England. McDouagh (1925) reported good results from the use of manganese butyrate (1–1.5 cc. of a 1 per cent solution) in boils, carbuncles and a variety of infectious conditions. Although some of the experimental work may indicate that manganese stimulates the self defense mechanism of the organism, more experimental and clinical data must be submitted before its use for such purposes can be accepted.

SUMMARY

From the data presented in the literature it appears that manganese is a regular constituent of plant and animal tissues and that it has important metabolic functions in both.

In animals it is mainly stored in the liver and in the kidneys.

Its absorption from the intestinal tract is very slow and incomplete under normal conditions. The degree of absorption depends upon the acidity of the gastric juice and the solubility of the manganese compounds.

The excretion takes place in the colon, partly also with the bile and to a moderate extent with the urine.

With oral administration to animals toxic symptoms are only observed with large doses which lead to gastric disturbances. With subcutaneous injections central nervous symptoms are reported and with intravenous administration circulatory disturbances are the most characteristic symptoms. Manganese appears to stimulate the growth of young animals, its effect on the generation of blood needs further study. Animals treated with manganese compounds in different ways show degeneration of the liver and other organs, later also degenerative changes of the central nervous system which vary as to their extent and location with different species.

Workmen exposed to the dust of manganese compounds may develop manganese poisoning. This resembles in its clinical picture progressive lenticular degeneration (Wilson's disease). It appears possible that there is a primary affection of the liver and that the symptoms from the central nervous system may be secondary.

The toxicity of manganese varies with different salts. Therapeutically manganese compounds have been advocated in a great variety of conditions, but further studies and clinical observations appear necessary before these can be accepted.

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THE SPECIFIC DYNAMIC ACTION OF FOOD

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In the present review no attempt has been made to cover the historical development of the subject nor to review the outstanding theories which guided the experimental efforts of a past generation of workers. This aspect of the subject will be found in the numerous articles by Graham Lusk and in his inimitable book, *The Science of Nutrition*. The present review aims to present briefly the present status of the problem and particularly to emphasize certain newer concepts and experimental evidence which are causing rapid changes in contemporary theories.

Attention should be called to three recent reviews which treat of certain phases of this problem, namely, those of Luck (1), Brody (2) and Lusk (3). The review by Lusk is particularly interesting because it was his last review article and in it he points out that recent experimental evidence necessitates radical changes in his well known theories on the cause of the specific dynamic action of protein and carbohydrate.

The term specific dynamic action was given by Rubner to the increase in heat production which follows the ingestion of any of the primary food materials or certain of their primary degradation products. Modern experimental work is showing quite clearly that the term should be considered as generic rather than as specific, in that the total rise in heat production which follows the ingestion of any of the primary food substances may be the result of several rather than of one specific process.

The basal heat production during fasting is largely the result of processes whereby food materials, stored in the body, are mobilized, chemically altered, the waste products excreted, and the remaining portions utilized by the cells. Therefore in a broad sense the basal heat production during fasting is to a great extent the S. D. A. of endogenous or stored food materials. This was demonstrated in the interesting experiments of Richardson and Mason (4) who calculated the protein, carbohydrate and fat utilized in 24 hours during fasting and then gave this mixture of food materials in small amounts every two hours during fast-

ing and observed that this "replacement diet" caused practically no increase in basal heat production. This concept of S. D. A. is an important one which would bear further investigation.

A. THE S.D.A. OF PROTEIN AND AMINO ACIDS. The specific dynamic action of protein in normally nourished animals can very likely be assumed to be due to the sum of the separate effects of the constituent amino acids (5); therefore certain phases of the problem can be considerably simplified by studying single amino acids or combinations of several amino acids. The S.D.A. of proteins and of single amino acids appears to be similar in normally nourished animals but not in fasting animals in which complete proteins may be stored and exert little or no S.D.A. (6, 7, 8), while single amino acids (alanine and glycine) have a definitely greater S.D.A. in the fasting than in the normally nourished animal (9).

When single amino acids are administered to normally nourished animals it is found that all do not have the same S.D.A.; alanine and glycine are practically the same (10, 11), phenylalanine and tyrosine have a much greater effect (10, 12) while valine and histidine are reported to have no effect (5, 12). The much debated effect of glutamic acid will be referred to later.

With regard to the cause of the S.D.A. of amino acids the following negations deserve special emphasis:

1. The S.D.A. is not due to the direct combustion of the amino acid per se and liberation of its energy content as extra heat. The classic experiments of Lusk in which he administered glycine and alanine to phlorhizinized dogs and recovered the entire energy content of the amino acids in the urine as "extra" glucose and urea but observed a high S.D.A. definitely established this point. The experiments of Wilhelmj and Bollman (10) also indicated that this explanation is not valid since they found that the S.D.A. of alanine and glycine often greatly exceeded (1½ to 3 times) the energy content of the amount of amino acid deaminized during the experimental period.

2. The S.D.A. is not due to direct stimulation of the tissues by the unchanged amino acids. Lusk presented evidence against this hypothesis years ago and it was definitely excluded by the studies of Wilhelmj, Bollman and Mann (13), in which they found that intravenously administered amino acids (alanine and glycine) caused no elevation of heat production in hepatectomized dogs.

3. The S.D.A. is not influenced by the work of the digestive glands, intestinal movements, work of absorption from the intestine, etc. This

has been definitely proven for the amino acids glycine and alanine by the fact that the effect is the same whether they be administered intravenously, orally or subcutaneously (14, 15, 16, 17). Wilhelmj, Bollman and Mann (17) also observed that the S.D.A. was not altered by the rate of intravenous injection and confirmed the observation of Lusk (18) that the effect was the same with the sodium salt as with the unneutralized amino acid. The work of Benedict and Emmes (19) confirmed the observation of Rubner that violent intestinal peristalsis caused negligible increases in heat production.

Regarding the similarity of the S.D.A. with oral and subcutaneous administration, Lewis and Luck (20), found that in rats subcutaneous and oral administration gave the same S.D.A. only when small amounts were administered. When larger doses were given they found that subcutaneous administration gave a much lower S.D.A. and that when 2 grams per kilo were given subcutaneously there was no S.D.A. The higher doses (2 grams per kilo) were violently toxic and often caused death, so that this observation is very difficult to interpret and is possibly not comparable to experiments with smaller non-toxic doses.

I. The site of the extra heat production. Mann, Wilhelmj and Bollman (21) studied the effect of intravenous administration of the amino acids alanine and glycine in hepatectomized dogs and found no increase in heat production. Wilhelmj, Bollman and Mann (13) repeated these experiments in another series of animals and definitely established the fact that the S.D.A. of alanine and glycine is abolished by removal of the liver. Bollman, Mann and Magath (22) had previously shown that when amino acids were administered to hepatectomized dogs no deamination or urea formation occurred and that the entire quantity of amino acid could be recovered in the blood, urine and muscle. The experiments of Wilhelmj, Bollman and Mann do not necessarily prove that the S.D.A. of amino acids normally results from the process of deamination and urea formation per se, nor do they establish the fact that the liver is the site of the extra heat production.

Perfusion of isolated tissues has, with one exception, appeared to confirm the above findings. Bornstein (23, 24), Bornstein and Roese (25), Nothhass and Never (26) found that when the amino acid glycine was added to blood perfused through the liver, there was a marked increase in oxygen consumption, accompanied by a decrease in amino nitrogen and an increase in urea nitrogen; when, however, glycine was added to blood perfused through the muscles of the extremities no increase in oxygen consumption occurred. Rapport and Katz (27),

however, found that the oxygen consumption of isolated perfused muscles gradually decreased during the experimental period but when glycine was added to the perfusing blood, there was an increase instead of the usual decrease. They believe that the S.D.A. of glycine depends upon direct stimulation of the tissues. It should be pointed out that their results may be non-specific and depend upon osmotic pressure effects (vide infra) since the amount of glycine added to the blood was rather larger.

The interesting experiments of Dock (28) point to the liver and not the muscles as the actual site of the extra heat production constituting the S.D.A. of casein. The oxygen consumption of rats on a high casein diet was 35 per cent higher than control animals. The oxygen consumption of the hind quarters, determined by ligation of the aorta below the renal arteries, was only 8 per cent greater in the casein fed group, while the oxygen consumption of the abdominal viscera was 141 per cent greater in the casein fed group. Dock believed the liver to be exclusively responsible for the S.D.A. of casein.

While it is quite evident that the liver is primarily responsible for the S.D.A. of amino acids and protein, the actual site of the extra heat production is still doubtful. The experiments of Dock seem to indicate that the extra heat is actually produced in the liver; Bornstein and Roese, however, placed the liver in a muscle perfusion circuit and found that when the liver was present, amino acids added to the perfusing blood increased the oxygen consumption of the muscles as well as of the liver. They suggest that a hormone is produced in the liver by the amino acid, which then causes an increase in the oxygen consumption of the muscles.

II. *Is the S.D.A. of amino acids associated with deamination and urea formation or with the metabolism of the deaminized residue?* This question is of major importance in the problem of S.D.A. Lusk believed that deamination and urea formation take place without appreciable energy expenditure and that the real increase in heat production results from the metabolism of the deaminized residue. In his last review article (3) he expressed the opinion that the S.D.A. of alanine and glycine represents the energy necessary to convert the deaminized remainder into glucose.

That deamination and urea formation are accomplished without appreciable energy expenditure appeared very probable in view of Lusk's observation (29) that glutamic acid was deaminized and the nitrogen excreted as urea without appreciable increase in heat production. Later

Atkinson and Lusk (30) made the same observation using aspartic acid. In 1930 Chambers and Lusk (31) again failed to demonstrate a definite S.D.A. for glutamic acid. Aub, Everett and Fine (32) also found that there was no increase in heat production when glutamic acid was administered intravenously to decerebrate cats. Other workers have been unable to confirm these very important observations. Grafe (33) was one of the earliest workers to insist that glutamic acid has a very definite S.D.A. Rapport and Beard (34) reported that both glutamic and aspartic acids have a very definite S.D.A. Lundsgaard (15, 36) found that glutamic acid has a S.D.A. practically as high as glycine. I am indebted to Prof. James Murray Luck (35) for a report of his recent unpublished studies in which he likewise finds that glutamic acid has a S.D.A. which is comparable to that of glycine. He finds, however, that the S.D.A. of glutamic acid is profoundly influenced by several hitherto unrecognized factors which may explain, in part, the contradictory results of other workers. It is difficult to explain Lusk's repeated negative results but it is now necessary to state that glutamic acid has a very definite S.D.A. and to thus remove the main evidence for the statement that deamination and urea formation take place without appreciable energy expenditure.

Several recent investigators have presented some very interesting and important data which appear to link the S.D.A. with the nitrogen fraction rather than with the nitrogen free residue of amino acids. Lundsgaard (15, 36) found that sodium acetate produces only a very slight elevation of heat production while ammonium acetate produces a very marked increase; similarly, sodium lactate has only a very slight effect while ammonium lactate produces an increase in heat production which is equal to that of an equivalent quantity of alanine. He stressed the fact that while glycine has a very high S.D.A. sodium acetate has very little. Further studies by Lundsgaard (15) showed that the administration of most ammonium salts causes a large rise in heat production, ammonium chloride for instance was found to cause an increase in heat production, per gram of nitrogen given, which was equivalent to that of alanine. He also found that the S.D.A. of alanine and glycine is equivalent to their nitrogen content and is equal to about 8 calories per gram of nitrogen given. He concluded that the S.D.A. of protein and amino acid is associated with the nitrogen fraction and is due either to urea formation or to the stimulating effect of the ammonia primarily formed. It should be recalled that Grafe (33) likewise found a marked

increase in heat production following the administration of certain ammonium salts, especially ammonium chloride.

The recent analysis by Borsook and Winegarden (37) shows that there is a close correlation between the excretion of urinary nitrogen and the S.D.A. of protein and amino acids and they state "that neither the direct experimental evidence nor the considerations of energy relations supports the view that the S.D.A. of protein is necessarily due to the conversion of the deaminized fractions into glucose."

Terroine, Bonnet and Zagami (38) pointed out that both lactic and pyruvic acids are possible intermediary products in the metabolism of alanine; they found, however, that when the sodium salts were administered to rabbits there was practically no increase in heat production, from this they concluded that the S.D.A. of alanine is not associated with the nitrogen-free residue but with the nitrogen fraction. Zagami (39) pointed out that both malic and citric acids are converted to glucose in the diabetic animal but found that administration of the sodium salts to rabbits caused no increase in heat production from which he concluded that the S.D.A. of the sugar forming amino acids is not associated with the transformation of the nitrogen-free residue to glucose.

Terroine, Trautman, Bonnet and Jacquet (40) studied the energy transformation of a species of mold when grown on media containing certain substances as the sole source of growth. On glucose they found the energy loss very small but on certain amino acids the energy loss was very large (average 35 per cent). They expressed the opinion that the loss of energy was associated with the process of deamination and not with the transformation of the nitrogen-free residue to glucose. Terroine and Bonnet (41) tested this hypothesis by studying the same mold on a media containing malic, citric and tartaric acids and found that the loss of energy was only slightly greater than when grown on glucose. From these studies they concluded that the S.D.A. of protein and amino acids is associated with deamination and not with conversoin of the nitrogen-free residue to glucose.

Lusk (42) likewise observed that glycollic and lactic acids do not give a S.D.A. at all comparable to the corresponding amino acids glycine and alanine but believed that this did not exclude the possibility of their acting as powerful stimuli "if they were liberated at the surface or within the cell as the result of deamination."

The finding of Wilhelmj, Bollman and Mann (13) that the S.D.A. of

amino acids is absent in the hepatectomized dog, in which deamination does not occur, while it by no means proves it, is at least in harmony with the theory that the S.D.A. of amino acids is associated with the process of deamination and urea formation and not with the conversion of the deaminized residue to glucose.

III. *Chemical and thermochemical considerations.* Before considering this phase of the problem it is necessary to emphasize that no chemical or thermochemical equation has yet been written which is in harmony with experimental findings.

As stated above, Lusk believed that the S.D.A. of alanine and glycine could be explained on the basis of transformation to glucose and urea. In 1926 Adams (43) calculated from the second and third laws of thermodynamics that in order to transform glycine and alanine to glucose and urea according to the equations of Lusk would necessitate the expenditure of certain quantities of energy, whereas the same calculations applied to glutamic acid showed that the change to glucose and urea could be accomplished without the expenditure of energy. Chambers and Lusk (31) expressed the opinion that "these calculations of Adams, explain in part, if not wholly, the S.D.A. of protein in the simplest way." According to the calculations of Adams it would require 0.871 calorie to change one gram of glycine to glucose and urea whereas for alanine the value would be 0.337 calorie. When these values are expressed as the energy necessary to transform one millimole of the amino acids the energy amounts to 0.067 calorie for glycine and 0.031 calorie for alanine. Wilhelmj (11) analyzed a series of 42 experiments with alanine and glycine published from the laboratory of Wilhelmj and from that of Lusk and found that the values obtained in both laboratories were practically identical when the S.D.A. was expressed as calories of extra heat per millimole of amino acid deaminized. For each millimole of amino acid deaminized the S.D.A. of alanine and glycine was practically identical, the average value for glycine being 0.20 calorie and for alanine 0.19 calorie. Wilhelmj pointed out that the actual S.D.A. of glycine is thus approximately three times, while that for alanine is approximately 6 times the value calculated by Adams. While it is true that energy transformations in the body can never be 100 per cent efficient, this difference between the calculated and the observed S.D.A. of alanine and glycine is far too great to be explained simply by assuming a low efficiency in the process of transformation.

The calculations of Adams have been critized by Borsook and Winegarden (37) who believed that insufficient data are available, at present,

to make these calculations according to the second and third laws of thermodynamics. Their calculations according to the first law of thermodynamics, however, gave practically identical values.

The equations of Lusk and the calculations of Adams are based on the assumption that the normal metabolic pathway for alanine and glycine is to glucose and urea. While it is true that these amino acids are transformed to glucose in the phlorhizinized or diabetic dog and that a S.D.A. occurs under these conditions, which is comparable to that in the normal dog, it does not necessarily follow that this is the usual metabolic change in normal animals. Two recent, independent groups of workers have investigated this question by studying glycogen formation in rats after administration of certain amino acids; with alanine, glycine and glutamic acid their findings were identical. Wilson and Lewis (44) found definite evidence of glycogen formation after administration of alanine but only slight evidence after glutamic acid. Greisheimer and Arny (45), likewise found definite evidence of glycogen formation after alanine but practically none after glutamic acid and glycine. Hence the assumption that these three amino acids follow the same metabolic pathway and are converted to glucose is open to doubt.

That there is some relationship between the S.D.A. of protein and amino acids and carbohydrate metabolism appears quite likely. Following the intravenous administration of alanine and glycine there is normally a rise in blood sugar (46) which according to Nord (47) is abolished by adrenalectomy. When amino acids or protein are administered to normal dogs the respiratory quotient usually rises above the basal value and is often above the theoretical quotient for protein or the particular amino acid administered. That this elevation of the R.Q. is not due to displacement of carbon dioxide from the blood by the carboxyl group of the amino acid is suggested by the work of Chanutin (48) who found that the carbon dioxide combining power of the blood is usually materially increased after the administration of meat or amino acids. Taistra (49) also showed that the S.D.A. is not dependent upon the level of the alkaline reserve of the blood plasma. Wilhelmj, Bollman, and Mann (17) studied the response of dogs to intravenously administered glycine. They found that the rise in R.Q. was practically as great when the amino acid solution was neutralized to a pH of 7.4 with tenth normal sodium hydroxide as when the unneutralized solution was given and that the S.D.A. was practically the same in both instances; from this they concluded that the rise in R.Q. following administration of amino acids or meat, is of true metabolic significance. Wilhelmj and

Mann (9, 50) found that the behavior of R.Q. following the intravenous administration of alanine or glycine was dependent upon the nutritional condition of the animal. When the animal was on a standard, normal diet there was usually a definite rise in R.Q., while during total fasting of several days' duration, the S.D.A. occurred with no rise in quotient, the basal value of 0.70 being maintained throughout. After several days on a very high carbohydrate diet, following a period of fasting, the injection of the same quantity of amino acid was followed by a much greater rise in R.Q. than in the normal animal, the quotient often rising to 1.0 or above, the height apparently depending on the amount of carbohydrate in the previous diet. Lusk (51) likewise observed that in the fasting phlorhizinized dog the R.Q. after alanine and glycine would usually remain at approximately 0.70. These observations show that the extra heat production constituting the S.D.A. of the amino acids alanine and glycine may come primarily from carbohydrate or from fat depending upon the nutritional condition of the animal.

Kiech and Luck (52) have recently reported some very interesting and important studies which may be instrumental in opening a new approach to the question of the intermediary steps in the metabolism of alanine and glycine. These observers used rats and following the administration of the amino acid analyzed the entire carcass and the excreta for amino and urea nitrogen. With d-l alanine they found that there was an initial rapid fall in amino nitrogen but that little more than 50 per cent of the nitrogen was converted to urea. With subcutaneous administration of glycine there was also an initial lag in urea formation which extended over 6 to 8 hours, followed by a second period in which urea formation exceeded that which could have resulted from deamination of glycine. They postulate that there is a formation of nitrogenous intermediates from the amino acids, the nitrogen of which does not react with nitrous acid but which is ultimately converted to urea.

Luck and Amsden (53) using dogs, followed the excretion of urea and amino acid nitrogen and total sulfates for long periods following the parenteral injection of glycine, alanine and glutamic acid. For a period of $7\frac{1}{2}$ hours after injection of glycine and glutamic acid, the urea formation was greatly in excess of that which should have come from the amino acid, while for alanine it was far below. In all cases the injection of the amino acid was followed by a 30 to 90 per cent increase in the excretion of total sulfate. They concluded that there is a stimulation of endogenous protein catabolism following administration of these amino acids.

IV. *The possible rôle of osmotic pressure changes in the blood and tissue fluids.* Following the ingestion and during the metabolism of meat and amino acids osmotic pressure changes occur in the blood and tissue fluids. That these changes may be responsible for at least a part of the increase in heat production is suggested by several facts. Wilhelmj, Bollman and Mann (17) studied the S.D.A. of 8.18 grams of glycine, administered intravenously. They then injected saline solutions which were approximately isotonic with the glycine solutions and observed increases in heat production which were equivalent to from 30 to 50 per cent of the S.D.A. of the glycine solution. When physiologic saline was given intravenously no increase in heat production occurred. Glucose solutions approximately isotonic with the glycine solution were followed by increases in heat production which were very much less than those following the glycine or saline solutions. They suggested that the rapid withdrawal of fluid from the tissues to readjust the osmotic pressure of the hypertonic saline solutions was the factor responsible for the increase in heat production. Castex and Schteingast (54) injected saline solutions subcutaneously in human subjects and observed definite elevations of basal metabolism ranging from 4 to 19 per cent and averaging 11 per cent. Lublin (55) administered 7.6 grams of sodium chloride by mouth and observed an elevation of metabolism amounting to 8 per cent. Lundsgaard (36) administered 1 to 1½ gram of sodium chloride by mouth and observed increases in basal metabolism ranging from 0.8 to 8 per cent. Several recent workers have reported small but definite increases in heat production following oral administration of water. Grollman (56) observed increases from 5 to 10 per cent following the ingestion of water, but no increase following the ingestion of a similar amount of physiologic saline or Locke's solution. Carpenter and Fox (57) likewise reported small but definite increases in heat production following oral administration of water.

V. *The possible rôle of the work of the kidneys.* Lusk (58) administered urea in amounts which might have come from the metabolism of large quantities of meat and found that there was no increase in heat production even when urea was being eliminated at the rate of one gram per hour. He likewise observed that oral administration of sodium chloride was without effect. From this he concluded that kidney activity was not a factor in the S.D.A. of protein or amino acids. Aub, Everett and Fine (32) likewise observed practically no increase in heat production when urea was administered in decerebrate cats.

This question has been recently reopened and several investigators

have presented evidence which seems to indicate that the work of the kidney may be a factor of importance in the S.D.A. of proteins and amino acids. Borsook and Winegarden (59) administered from 15 to 20 grams of urea to human subjects and observed very definite increases in basal metabolism. They found that the extra heat production per gram of extra nitrogen eliminated varied from 6 to 11 calories. They found that the "efficiency" of the kidney is low, probably not greater than 1 to 2 per cent. In a second paper (37) they analyzed the specific dynamic action of protein and amino acids from the standpoint of the energy cost of the excretion of urine and found that a close correlation exists between the S.D.A. of protein and amino acids and the excretion of the "extra" urinary nitrogen, and that from 25 to 60 per cent of the S.D.A. may be due to the work imposed on the kidney. They state that "the remainder of the S.D.A. of protein or amino acids is due to the metabolism of the nitrogen and the carbon, though it is not possible, from the evidence, to estimate the proportion for which each is responsible." Thus according to their hypothesis S.D.A. is due to at least two distinct processes.

In sharp contrast to the conclusions of Borsook and Winegarden is the observation of Strieck (60) who found that the S.D.A. of meat, in dogs, was approximately the same after removal of the kidneys as before; his data, however, are not entirely satisfactory and strict comparisons are difficult.

Lublin (55) administered water, urea and sodium chloride and observed increases in heat production which paralleled the excretion and diuresis and were attributed by him to the work of the kidney. Sixty grams of urea in 500 cc. of water caused an elevation of heat production of from 8 to 12 per cent, while frequent and repeated administration of water following the urea caused further elevation of heat production up to 20 per cent. Several of the findings discussed in the previous section (osmotic pressure) have likewise been attributed to the work of the kidney.

This interesting problem could be very definitely settled by a careful study of the S.D.A. of intravenously administered amino acids in properly trained dogs before and after nephrectomy.

VI. *The influence of the hypophysis and other glands of internal secretion.* Beginning with the studies of Plaut (61) several investigators have reported that the S.D.A. of protein is either greatly lowered or abolished in human subjects with pituitary disease or in animals after

removal of the pituitary gland. Recently Goldzieher and Gordon (62) reported a study on human cases, showing a greatly lowered S.D.A. in pituitary disease. Foster and Smith (63) reported that hypophysectomy in rats caused a disappearance of the S.D.A. of glycine. However, there is gradually accumulating a rather formidable array of careful work, both clinical and experimental, which throws great doubt on these previous findings. Fulton and Cushing (64) and Johnson (65) report that the S.D.A. of protein is normal in human cases of pituitary disease. Daggs and Eaton (66), Artundo (67), and Gaebler (68), all report normal S.D.A. in dogs after removal of the pituitary gland.

DuBois (69) found the S.D.A. of protein and glucose practically within normal limits in exophthalmic goiter and hypothyroidism.

Nord and Deuel (14) found a normal S.D.A. in dogs following adrenalectomy.

VII. *The influence of the state of nutrition.* Wilhelmj and Mann (9, 50) studied the S.D.A. of intravenously administered glycine, alanine and phenylalanine in the same dog during a period of complete fasting of approximately two weeks, and during realimentation with the standard diet or with an almost exclusive carbohydrate diet (Karo syrup and cracker meal). They found that the S.D.A. of glycine and alanine was greatly increased during fasting, while the amount of amino acid deaminized, during the four hour experimental period, was greatly reduced. In some, but not all, animals there was evidence of a toxic reaction (vomiting) following administration of alanine or glycine during fasting. In spite of the greatly increased S.D.A. the respiratory quotient did not rise above the fasting value of 0.69 to 0.70. The S.D.A. of phenylalanine was not changed during a similar period of fasting. The greater S.D.A. and decreased amount of amino acid deaminized are undoubtedly related phenomena and suggest the possibility that a synthesis of more complex nitrogen containing compounds may occur, which are retained in the body for at least a short time. The greater S.D.A. during fasting would then represent the extra energy required for these synthetic processes. Other possibilities are discussed in the original paper. The studies of Kiech and Luck (52) seem to give direct evidence of the synthesis of nitrogen containing intermediary products from amino acids and these synthetic processes may be increased during fasting. When the animals were returned to the standard diet there was a gradual return of the S.D.A. to normal. In a later paper Wilhelmj and Mann (50) found that if the animals were fasted for two

weeks and then given an almost exclusive carbohydrate diet, the S.D.A. was greatly reduced, even below the normal level, and that the toxic manifestations were abolished.

The experiments of Wilhelmj and Mann represent extreme conditions but there is much evidence which suggests that the level or plane of nutrition may alter the S.D.A. of foods. Mason (70) found evidence of an abnormal S.D.A. for either protein, carbohydrate or fat in cases of undernutrition, with gain in weight when the offending food material was eliminated from the diet.

The old question of whether fat and thin people have a different value for the S.D.A. of food continues to end in contradictions. Wang and Strause (71) find a high value for the S.D.A. of protein in thin people and a low value in fat people. Strang and McClugage (72) in a very excellent paper deny that this difference exists.

VIII. *The manner of expressing and comparing S.D.A.* Much of the confusion and contradiction found in the literature is undoubtedly due to the fact that there is no uniform or generally accepted manner of expressing the S.D.A. of protein and amino acids and hence no standards whereby comparisons can be made. Wilhelmj and Bollman (10) discussed this question and showed that the S.D.A. of amino acids could best be expressed as the calories of extra heat per millimole of amino acid deaminized. When this manner of expression was used they found that the S.D.A. of alanine and glycine were practically identical while that of phenylalanine was much greater. Wilhelmj (11) recently analyzed a series of experiments published by Wilhelmj, by Lusk and by Rapport. Forty-two experiments from the laboratory of Wilhelmj and that of Lusk gave an average value of 0.20 calorie per millimole of amino acid deaminized; 35 experiments with glycine gave an average of 0.20 calorie while 7 with alanine gave an average value of 0.19 calorie. The amino acids were administered both orally and intravenously and the amounts given varied from 3.69 to 30 grams and gave total S.D.A. ranging from 5 to 34 calories; the dogs used varied in size from 6.9 to 18.2 kgm. with basal levels of heat production ranging from 11.7 to 21.6 calories per hour. The amounts of amino acid deaminized varied from 20 to 184 millimoles. The S.D.A. per millimole of amino acid deaminized varied between 0.16 and 0.27 calorie. In view of the numerous variable factors the constancy of the S.D.A. per millimole of amino acid deaminized is remarkable.

Weis and Rapport (16) found that when increasing amounts of meat were given to dogs the S.D.A. was not a linear function of the amount

given but of the amount metabolized. When S.D.A. was expressed as $\frac{\text{S.D.A.}}{\text{100 cal. meat metabolized}}$ they found rather constant values ranging from 32 to 38 calories in their own experiments and also in some experiments of Lusk which were recalculated and expressed in this manner.

Lewis and Luck (20) using rats were led to the conclusion "that no simple relationship exists between the quantity of glycine given and the increase in metabolism induced thereby. Any theory which seeks to explain the S.D.A. of glycine (or other amino acid) by postulating that the increased caloric production is a fixed and definite quantity per millimole of glycine, must be of doubtful validity. It is clear that various factors are involved (mode of administration, quantity given, secondary effects due to toxicity, nutritional state) which do not permit such a simple hypothesis." It should also be remembered that Wilhelmj and Mann (9, 50) were able to produce marked variations in the S.D.A. per millimole of amino acid deaminized by extreme changes in the nutritional condition of the animal.

In spite of the statement of Lewis and Luck and the findings of Wilhelmj and Mann it appears quite likely that standard normal values for the S.D.A. of protein and amino acid could be worked out on the basis of the amount of amino acid or protein deaminized which would give a fairly accurate basis for comparison.

B. Specific dynamic action of glucose and carbohydrate. I. *The site of the extra heat production.* In their studies on S.D.A. following hepatectomy Wilhelmj, Bollman and Mann (13) showed that whereas the S.D.A. of amino acids is abolished by liver removal, the S.D.A. of intravenously administered glucose is not only present but is usually greater than in the normal animal. They suggested that the greater S.D.A. after liver removal is possibly due to the fact that normally a certain amount of the glucose is held by the liver whereas after liver removal all of the injected glucose reaches the particular tissues in which it causes an elevation in heat production. From these studies it is obvious that the S.D.A. of glucose is not dependent upon the liver.

II. *The cause of the S.D.A. of glucose and carbohydrate.* For many years the most generally accepted explanation of the S.D.A. of glucose and carbohydrate was that of Lusk, called by him the "Plethora Theory." According to this theory the increase in heat production following the ingestion of glucose was due to combustion of the glucose by the cells, the increased combustion being due to the fact that an excess amount of glucose was brought to the cells by the blood stream, hence a plethora of glucose molecules existed.

In his last review article Lusk (3) cited evidence against the plethora theory and stated that it must be modified. Even before this time, however, several investigators had presented evidence which was difficult to reconcile with the plethora theory. Mason (73) found that following the administration of glucose there was little relationship between the height of the blood sugar, the rise in R.Q. and the S.D.A. of glucose in diabetics. Rabinowitz (74) found that when glucose was administered to patients with normal carbohydrate tolerance (renal glycosuria) there was practically no relationship between the height of the blood sugar, the rise in R.Q. and the S.D.A. Deuel (75) pointed out that when 75 grams of glucose were administered to a normal human subject there was frequently little relationship between the time of the maximal heat production and the highest R.Q. and that there was no relationship between the total carbohydrate metabolism and the S.D.A. or between the "extra" carbohydrate calories and the total S.D.A. He pointed out that a rise in R.Q. even when it is below 1.0 may be the result of the conversion of glucose to fat and not the oxidation of glucose.

The findings of Baur (76), were very instrumental in Lusk's change of attitude. Baur administered 85 grams of glucose to a human subject on the first day of fasting and found only a very slight increase in heat production amounting to 4 per cent during the first two hours, while during the third hour the heat production dropped below the basal value. On the fifth day of fasting the subject was given very strenuous exercise to reduce the glycogen stores, following this the administration of 85 grams of glucose resulted in an enormous S.D.A. of +21, +12, and +10 per cent during the three hours after administration. Baur interpreted these findings as signifying that the S.D.A. of glucose represents not combustion of glucose, but the chemical energy necessary to transform glucose to glycogen. Dann and Chambers (77), in Lusk's laboratory, studied the effect of administering 50 grams of glucose to dogs following fasting periods of from 19 to 31 days. The first experiment following the fasting period was characterized by a marked hyperglycemia and glycosuria with little rise in R.Q. but a marked S.D.A. Only a very small amount of the glucose administered could be accounted for by oxidation and excretion. They found no relationship between S.D.A. and the amount of glucose oxidized or between the height of the blood sugar and the glucose oxidized. They concluded that the S.D.A. of glucose may not be due to a plethora with oxidation of glucose but to the synthesis of glucose to glycogen. One of the cases studied by Rabinowitz (74) was a patient with renal glycosuria who through fear of

diabetes had lived upon a low carbohydrate diet for weeks. Following the administration of 100 grams of glucose, there was a slight fall in R.Q. (from 0.73 to 0.71) but a marked rise in heat production from 84 to 92 calories per hour. This observation like those of Baur and Dann and Chambers suggests that, at least under conditions of fasting with reduced glycogen stores, the S.D.A. of glucose may represent the energy necessary to synthesize glucose to glycogen.

The studies of Carpenter and Lee (78) also demonstrate a lack of correspondence between the rise in R.Q. and the S.D.A. of carbohydrate. Edwards, Margaria and Dill (79) also noted that there was often little relationship between the blood sugar, the S.D.A. and the respiratory quotient following administration of glucose.

In connection with the above studies it will not be out of place to call attention to the fact that a rise in R.Q. may not necessarily mean combustion of glucose and that to consider the R.Q. as a combustion quotient only is quite possibly a serious error. The excellent analysis of the significance of the R.Q. by Cathcart and Markowitz (80), made this obvious.

III. *The behavior of certain trioses which are converted to glucose.* 1. Glycerol is completely converted to glucose in the diabetic and phlorhizinized animal but Chambers and Deuel (81) found that it exerted no S.D.A., hence it would appear that the conversion of glycerol to glucose does not require the expenditure of energy.

2. Dihydroxyacetone which is likewise converted to glucose in the phlorhizinized dog (82) behaves quite differently from glycerol. Mason (83) found that there was a marked rise in R.Q. and heat production following the administration of dihydroxyacetone to normal men. The rise in heat production was greater than after a similar amount of glucose. Mason suggested that dihydroxyacetone or some of its intermediary products may be converted to fat. In diabetic subjects Mason (73) found that the height of the R.Q. after the administration of dihydroxyacetone depended upon the carbohydrate content of the previous diet, the higher the carbohydrate content of the diet the greater the rise. The dependence of the height of the R.Q. after administration of dihydroxyacetone upon the carbohydrate content of the previous diet is therefore similar to the behavior of the R.Q. after the administration of alanine and glycine as found by Wilhelmj and Mann (9, 50) and suggests that the previous diet may markedly influence the materials which are oxidized to supply the energy for certain intermediary chemical transformations.

C. THE S.D.A. OF FAT. The writer is not aware of any new material pertaining to the S.D.A. of fat, the "plethora thory" of Lusk being apparently undisputed. Unpublished experiments by Wilhelmj and Bollman, however, showed that there is frequently a marked discrepancy between the S.D.A. of fat and the behavior of the R.Q.

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THE PHYSIOLOGY OF THE HELMINTH PARASITES

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Although many species of invertebrate animals have been studied extensively in general and comparative physiology, the helminth parasites have not often been used for physiological studies. This has been true chiefly because it has not been possible to cultivate parasitic worms in artificial media, and indeed even difficult to maintain most of them alive outside the body of their host for any appreciable length of time. Consequently, the information regarding physiological processes in this group of animals is scanty and incomplete.

Knowledge concerning the physiology of the helminth parasites is of particular interest for several reasons: 1. They live in an unusual environment compared with other animals, an environment which is itself undergoing physiological processes. 2. The physiological reactions of the parasites, their nutrition, metabolism, and secretions, may have definite effects on their hosts. 3. An understanding of the physiological processes of helminths is of practical importance in the development of new and more efficient anthelmintics. The purpose of this paper is to summarize some of the experimental work on helminths which touches upon various fields of physiology.

The helminths belong in two phyla of the animal kingdom: 1, Platyhelminthes, or flatworms, including the flukes and tapeworms; and 2, Nemathelminthes or round worms. In spite of the fact that all helminths belong to approximately the same order of life, they are in no sense a homogeneous group but exhibit wide differences in structure, life history and habitat. They are very variable in size, ranging from 1 mm. or less in length for the smaller species to a length of over 30 feet for the larger tapeworms. In general, most of them possess definite nervous, digestive, muscular, reproductive, and excretory systems. They lack, however, definite circulatory and respiratory systems. Helminths, either in the larval or adult stage, may parasitize nearly every organ and tissue in the animal body, but the majority develop in the intestinal tracts of their hosts.

Comprehensive knowledge concerning the physiology of any of the groups of helminths, or even of any individual species, is not available. Consequently, in considering the different subjects to be reviewed, it will be necessary to skip about among the various groups of parasites, selecting the isolated facts that are known. On account of their large size and availability, the sheep liver fluke, *Fasciola hepatica*, and the large round worm of hogs, *Ascaris suum*, have been most widely studied and will be used most frequently as examples.

RESPIRATION AND METABOLISM. One of the first questions to arouse inquiry concerning the physiology of parasites was their respiration. It was known that the intestinal tract contained very little, if any, free oxygen and it was questioned whether intestinal parasites were aerobic or anaerobic in their metabolism. This problem has not been conclusively settled as yet, although the majority of workers on the subject have held the view that intestinal worms must possess an anaerobic metabolism.

As early as 1883, Bunge observed that cat ascarids could be kept alive several days in an oxygen-free environment. Weinland (1901; 1902; 1904a and 1904b) took up this problem in a series of investigations which have served as the authority in most text-books of comparative physiology. Working with *Ascaris suum* from hogs, he found that the worms lived in 1 per cent salt solution several days longer when kept under anaerobic conditions than when kept in the presence of air. Earlier work had already shown that ascaris possesses a comparatively high glycogen content, usually about 25 per cent of the dry weight of the worm. Weinland reasoned that the glycogen was the most probable source of energy for the worms, and undertook to study their metabolism during hunger under anaerobic conditions. He found that the glycogen content of the worms appreciably diminished during the experiment and that the principal products present in the external medium were carbon dioxide and volatile fatty acids, which were subsequently (1904a) identified as being chiefly valeric acid. These products were produced in approximately the same amounts under anaerobic as under aerobic conditions. According to his interpretation, the normal energy metabolism of ascaris was a fermentation process in which glycogen was anaerobically split into carbon dioxide and valeric acid, a type of fermentation similar to that shown by various yeasts and bacteria. In later work (1902), Weinland demonstrated that this anaerobic fermentation of glycogen also could take place in the juice pressed from

ground-up worms. In these latter experiments, antiseptics were used to inhibit bacterial action.

Flury (1912) also studied the excretory products of ascaris, and found that the usual products of protein metabolism, such as urea, uric acid, creatinin and purine bases, were never present. The substances present were mainly ammonium salts and fatty acids, such as caproic and butyric, but chiefly valeric. Flury reported that as much as from 0.4 to 0.85 gram of fatty acids was formed daily per 100 grams of ascaris. These findings, in general, supported the results of Weinland.

More recently several workers have questioned whether ascaris is actually an anaerobic organism. Slater (1925) kept worms in oxygen-free salt solution at 37°C. and found that after a few hours the worms became inactive, but remained alive for as long as 6 days. If they were then exposed to air they became active. When the worms kept under anaerobiasis were continually stimulated to move by means of induction shocks, they usually died in from 24 to 48 hours, whereas those in containers exposed to air, and also continually stimulated, lived. Slater attempted to sterilize living worms and study their metabolic products in the absence of bacteria but did not succeed. He questioned whether valeric acid was a true metabolic product of ascaris and expressed the opinion that its presence was due to bacterial action. He concluded that ascaris, when kept at 37°C. and deprived of oxygen, has the power to reduce its metabolic rate to a very low value, and that this state of suspended animation had misled Weinland into believing that the organism was anaerobic.

Although it had been known that ascaris could survive just as well in the presence as in the absence of oxygen, Adam (1932) was the first to demonstrate quantitatively that the worms utilized oxygen from the surrounding medium. He did this both by the Winkler titration method for the oxygen content of the medium and also by keeping the worms in a Krogh respirometer. He concluded that the normal length of life of ascaris outside the body is very variable, from 4 to 22 days, that the presence of oxygen has no unfavorable influence on their length of life, and that oxygen consumption is not influenced by a preceding period without oxygen. Harwood and Brown (1933) have recently confirmed Adam's work. They found that female worms (*Ascaris suum*) utilized from 0.03 to 0.045 cc. of oxygen (reduced to standard conditions of temperature and pressure) per hour. Male worms under the same conditions utilized somewhat more oxygen, from 0.05 to 0.07 cc. per hour.

Recent work has brought to light information concerning the metabolism of certain other helminths which is, in general, similar to that known for ascaris. Weinland and v. Brand (1926) studied the sheep liver fluke, *Fasciola hepatica*, and came to the conclusion that, like ascaris, this species gains its energy through an anaerobic fermentation process in which glycogen is split into carbon dioxide and fatty acids. With this species, however, the fatty acids were of the higher, non-volatile type. They also found that the length of life and the rate of carbon dioxide production of the flukes was the same whether the medium was saturated with hydrogen, nitrogen, or carbon dioxide-free air, and from this finding concluded that oxygen was of no significance in their life processes.

Harnisch (1932) confirmed the observation of Weinland and v. Brand that the carbon dioxide production by *Fasciola hepatica* is the same under aerobic and anaerobic conditions. He also ascertained that under the former circumstances the flukes are able to take up oxygen from the surrounding medium, but found that there was no fixed relation between the amount of oxygen consumed and the amount of carbon dioxide given off. For partial pressures of oxygen varying from that of air down to 1 per cent of an atmosphere, the amount of oxygen consumed varied directly with the oxygen tension of the medium. He came to the conclusion that energy production in *Fasciola hepatica* is actually anaerobic and that the oxygen consumption is an unrelated process in which the oxygen perhaps serves to raise certain organic substances in the flukes to a higher stage of oxidation.

The same type of study has been made by v. Brand (1929; 1933) for one of the large tapeworm parasites of sheep, *Moniezia expansa*. The worms were kept for 6 hours in Ringer's solution under anaerobic conditions at 39°C. During this time there was a decrease in glycogen content amounting to 0.25 per cent of the fresh worm substance. The fat content remained constant, while the nitrogen loss amounted to only 0.06 per cent. The excretory products found in the external medium were chiefly carbon dioxide, higher fatty acids, lactic acid, and succinic acid. Quantitatively the metabolic process took place as follows: 1.00 gram of glycogen (the amount consumed by 100 grams of worm substance per 24 hours) furnished 0.44 gram of carbon dioxide and 0.40 gram of ether-soluble substance which was composed of 0.20 gram of higher fatty acids, 0.16 gram of lactic acid, and 0.04 gram of succinic acid.

Alt and Tischer (1931) also studied *Moniezia expansa* and confirmed

v. Brand's finding that fatty acids and lactic acid in almost equal quantities make up the majority of the acids produced in the metabolism of the worm. They found that the amount of acid formed was practically the same either in the presence of 95 per cent oxygen mixtures or in the absence of oxygen. They also studied oxygen consumption by the tapeworm, and reported that the initial oxygen consumption was about twice as great as the level assumed after 2 hours and maintained for from 2 to 3 hours longer. This type of curve is in general similar to that reported by Adam (1932) for the oxygen consumption of ascaris and that reported by Harnisch (1932) for *Fasciola hepatica.*

The same type of oxygen relationships reported for *Moniezia expansa* has recently been shown by Harnisch (1933) to hold for another cestode, *Triaenophorus nodulosus,* an intestinal parasite of fresh-water fish. He suggests the hypothesis that oxygen consumption by these parasites may represent the fulfillment of an oxygen debt arising from the accumulation of oxidizable substances produced during the anaerobic metabolism of the worms and not completely removed from the body. In the presence of oxygen, these products would be oxidized quickly or slowly depending on the partial pressure of oxygen in the medium, and furthermore an equilibrium would be reached between the production and oxidation of metabolic products. Harnisch (1933) also reports measurements of the oxygen consumption by *Ascaris suum* which support the view that this parasite is an anaerobic organism. Pieces 1 cm. long, taken from the anterior third of the worm, showed over a period of time a constant oxygen consumption, the amount of which was directly related to the partial pressure of oxygen. Finely divided worm material also, in general, showed this same relationship, although it was not as marked as for the larger pieces of worm. On the other hand, carbon dioxide production by the pieces of worm was the same in 0.8 per cent oxygen as in 21 per cent oxygen.

The foregoing papers furnish information concerning the metabolism of representatives of the three main groups of helminth parasites, namely, Trematoda, *Fasciola hepatica;* Cestoda, *Moniezia expansa;* and Nematoda, *Ascaris suum.* The facts, in general, are the same for all three species. When the worms are kept under anaerobic conditions, there is a measurable decrease in glycogen content, and carbon dioxide and fatty acids are found in the external medium. The amount of carbon dioxide and acid produced is approximately the same under anaerobic as under aerobic conditions. With ascaris the fatty acids are mainly lower volatile acids, with *Fasciola hepatica* they are higher non-volatile

acids, while with *Moniezia expansa*, in addition to the higher non-volatile acids, lactic acid and succinic acid are also formed. All three species take up oxygen when kept under aerobic conditions. The amount of oxygen consumed, either by whole worms or pieces of worms, in general, varies directly with the oxygen tension of the medium. The amount of oxygen consumed by the worms is not related to the amount of carbon dioxide given off. These facts have been interpreted by the majority of workers to mean that the metabolism of these helminths must be anaerobic, consisting of a fermentation of glycogen into carbon dioxide and fatty acids.

Another argument advanced to support the anaerobic metabolism of helminths is that their natural environment in the intestine contains practically no free oxygen. Definite evidence on this subject is very meager. Fries (1906) measured the composition of rectal gas discharges in man and found a very low oxygen content, from 0.7 per cent to 1.3 per cent. He says that the samples were collected "with only a minimal introduction of outside air," so it is possible that this amount may represent a contamination. Adam (1932) in questioning the hypothesis that ascaris is an anaerobic organism, called attention to the work of Long and Fenger (1917) who measured the oxygen content of intestinal gases in recently slaughtered hogs and found oxygen in amounts ranging from 1.2 per cent to 14.2 per cent. In answer to this observation, v. Brand and Weise (1932) made a series of measurements of the oxygen content of bile from the gall bladder of cattle, sheep and dogs, and of the contents of the small intestine of dogs, cattle, sheep and hogs. The determinations were made in a Van Slyke apparatus and only small traces of oxygen were found in the bile of the animals studied, the maximum content being 0.084 volume per cent (the amount in water in equilibrium with an atmosphere containing 3.5 per cent oxygen). The fluid contents of the intestine also showed oxygen to be either entirely lacking or present only in exceedingly small quantities. In dogs, cattle and sheep not enough gas was present in the small intestine to analyze. Only a minority of hogs contained large quantities of intestinal gas and in these animals v. Brand and Weise confirmed the observations of Long and Fenger. They considered the presence of the gas, however, to be due to exceptional circumstances, and probably explained by the fact that the animals must have swallowed large quantities of air at the time they were killed. They believed that under ordinary circumstances *Fasciola hepatica* and *Ascaris suum* must live in environments practically free from oxygen.

Most helminth parasites have a stage in their life history during which they exist outside the body of a host. Numerous observations indicate that during this free-living period, metabolic processes in the worm are undoubtedly aerobic. Hookworm eggs survive several weeks in the absence of oxygen (Looss, 1911), but do not develop. They are, however, apparently adjusted to utilize oxygen at very low tensions, being able to develop normally in water containing only 0.4 cc. of dissolved oxygen per liter, an amount at room temperature in equilibrium with an atmosphere containing 1.4 per cent oxygen (McCoy, 1930). The same sort of relationship of oxygen tension to development also holds true for the eggs of *Ascaris suum* (Brown, 1928). The relation of oxygen consumption to metabolic products in the free-living stages of helminths has not as yet been studied.

One of the chief criticisms which can be made of the studies of the metabolism of helminths under anaerobic conditions is that the action of bacteria in the formation of the fatty acid end products has not been excluded. This objection is difficult to surmount experimentally, for it is practically impossible to free the adult worms from bacteria without fatally injuring them. v. Brand (1933) cites the following arguments against the bacterial origin of the fatty acids. 1. The acids produced by the various worms studied (Ascaris, Fasciola, and Moniezia) are different. Since ascaris and Moniezia are both intestinal parasites, it seems remarkable that the excretory products of one are never found in the external medium of the other. 2. In individual experiments, the amount of excreted acid is constant. 3. Succinic acid, one of the metabolic products reported for Moniezia, has also been found in echinococcus fluid (Flössner, 1925), a medium which is presumably sterile. 4. v. Brand and Weinland (1924) were able to demonstrate morphologically the secretion of fat particles in the excretory tubules of *Fasciola hepatica*, and more recently Vogel and v. Brand (1933) showed that the appearance of the fat particles in the excretory system coincides with the beginning of the parasitic phase of life, that is, with the beginning of anaerobic metabolism. It was impossible, however, to identify chemically this secretion with the fatty acids found in the external medium.

Summary. Most of the experimental evidence available supports the view that the metabolic processes of helminths are anaerobic and consist of the fermentation of glycogen into carbon dioxide and fatty acids. Nevertheless, the possibility of aerobic metabolism with oxygen utilized at very low tensions does not seem to be entirely ruled out.

Carbohydrate, in the form of glycogen, is apparently the chief substance metabolized. Mueller (1929) on morphological grounds, believes that fat as well as carbohydrate may be utilized by ascaris. In any event, the usual products of protein metabolism, urea, uric acid and creatinin are not found.

NUTRITION. The actual substances utilized by helminths for food are not very well known. Tapeworms lack a mouth and digestive tract and must absorb all of their food through the surface of their body. This presumably is composed of substances already digested in the intestine and ready for absorption by the host, and probably consists mainly of sugars. Certain other groups of helminths, such as the Filaroidea and Trichuroidea, even though they possess an intestinal tract, apparently subsist on readily absorbed substances rather than on formed elements which would require digestion. Other helminths with digestive tracts (Trematoda and Nematoda), however, have frequently been observed to contain blood corpuscles and other host tissue cells.

Early workers believed that blood must form the principal food of the sheep liver fluke, *Fasciola hepatica*, and some workers even described them as sucking blood from small blood vessels. Müller (1923) collected flukes from the small bile ducts and examined the gut contents microscopically. He could not demonstrate red blood cells or hemoglobin, but found bile duct epithelium and leucocytes. Regarding the method of digestion, he says that digestion in trematodes has usually been considered to be intracellular because certain free-living forms, such as Planaria, show this type of digestion. Cytological study of the intestinal epithelial cells of *Fasciola hepatica*, however, showed that they were secretory rather than phagocytic, and he came to the conclusion that digestion in this species is extracellular and not intracellular.

Weinland and v. Brand (1926) examined over 12,000 flukes from 148 sheep livers, and could classify them into four groups depending on whether they were in the gall bladder and large bile ducts or in the small ducts in the liver, and on whether the ceca of the worms were full or empty. From differences in the glycogen, fat, and nitrogen content of the four groups of the flukes, they concluded that the worms fed in the small bile passages of the liver, migrated to the larger ducts and gall bladder, after a time became hungry, and then returned to the small ducts again to feed. Like Müller they were of the opinion that the flukes fed on tissue elements and inflammatory exudates rather than on blood.

It has been noticed that many helminths which are either wholly or

partially embedded in the host tissue cause a necrosis and liquefaction of the tissue in their immediate neighborhood. These tissue reactions have been extensively studied by Hoeppli (1927; 1929). In certain species of intestinal nematodes, Hoeppli interprets the liquefaction of the mucosa around the anterior end of the worm as a form of extra-intestinal digestion in which the tissue is digested by a secretion produced by the parasite. The liquefied tissue then serves as nourishment for the worm. In certain species of Physaloptera, Feng (1931) ascribes the formation of this liquefying secretion to esophageal glands in the worms, but the nature of the secretion has not as yet been determined. An interesting example of extra-intestinal digestion is described by Szidat (1929) for the trematode species, *Cotylurus cornutus*. This parasite has a large hold-fast organ with which it attaches to the intestinal mucosa of the host. The tissue in close contact with the hold-fast organ undergoes necrosis and liquefaction, and is apparently ingested as food through the oral opening of the worm. The literature in regard to extra-intestinal digestion by parasites is reviewed by Hoeppli (1933).

Some ingenious experiments intended to prove that *Trichinella spiralis* must derive its food from the intestinal mucosa of the host have recently been reported by Heller (1933). Meat containing encysted trichina larvae was enclosed in collodion sacs, and the sacs introduced into the small intestine of susceptible hosts (cats and rats). The meat was digested in from 6 to 8 hours, indicating that the sacs were permeable to digestive juice. In examinations made after from 1 to 3 days, none of the larvae in the sacs showed any sign of growth or development. Also, larvae enclosed in fine silk bags and thus kept from contact with the intestinal mucosa, did not develop. In other experiments, India ink was introduced into the intestine along with meat containing encysted trichinae. Although India ink particles were abundantly present in the intestinal contents of the host, none could be found inside the intestinal tracts of the developing worms. Sections of intestinal tissue, fixed while the host was still alive, demonstrated that even the youngest stages of developing trichinae penetrated the intestinal mucosa, thus affording additional evidence that the parasite must depend on host tissue for nourishment.

Certain intestinal nematodes belonging to the group Strongyloidea are well-known blood-suckers and apparently subsist mainly on the blood of their host. The hookworm, which is the best-known example of this group, also ingests portions of intestinal mucosa detached by

means of the teeth which arm the buccal cavity. Wells (1931) has recently made an interesting study of the blood-sucking activities of the dog hookworm, *Ancylostoma caninum*. He operated on infected dogs under anesthesia, opened a loop of the intestine without injuring the blood supply, and was able to observe directly the behavior of the worms on the intestinal mucosa. Under optimum conditions, the muscular esophagus of the worms made very rapid sucking movements, sometimes at a rate as high as 120 to 250 pulsations per minute. The sucking of blood went on almost continuously, and persisted even after the posterior part of the worm had been cut off. For the most part, the red blood cells passed through the intestine of the hookworms unharmed. By counting the number of corpuscles and measuring the size of the drops ejected through the anus, Wells estimated that on the average one worm would take up as much as 0.84 cc. of the host's blood per 24 hours. The blood apparently passed through the worm's intestine too rapidly for appreciable amounts to be digested. Consequently, the worms must utilize diffusible substances in the plasma for food. Wells also observed that the hookworms could absorb oxygen from the ingested blood and suggested that the blood-sucking activity might also serve an auxiliary respiratory function. In this connection, it may be pointed out that Harwood and Brown (1933) found that hookworms under aerobic conditions utilize oxygen, per gram of body weight, at a rate approximately ten times greater than ascaris, a parasite which lives in the lumen of the intestine and does not suck blood. The possibility of hookworms living under anaerobic conditions has not as yet been investigated.

Certain helminths possess free-living stages in their life cycles which feed and grow in the open in the same manner as free-living organisms. The food utilized by these free-living stages can be studied experimentally more readily than that of the parasitic stages. The author (McCoy, 1929) demonstrated that dog hookworm larvae, which ordinarily develop to the infective stage in some sort of fecal mixture, utilize living bacteria for food. Hookworm eggs were sterilized and introduced into agar cultures of various bacteria. The larvae hatched and grew to the infective stage in the normal time of 5 or 6 days. They would not grow, however, in sterile feces, nor in cultures that contained heat-killed bacteria. These observations have been extended by La Page (1933) to include larvae of various other species of parasitic nematodes.

Summary. In general, the food utilized by helminths is composed

of readily absorbed substances present in the intestinal tract or tissues of the host. Certain species apparently secrete substances which liquefy the host tissue in the immediate vicinity of the worm so that it may be more readily ingested. This process has been described as extra-intestinal digestion. Other helminths, such as the blood and liver flukes, and members of the nematode group, Strongyloidea, may regularly contain blood corpuscles or other host tissue elements. Hookworms ingest relatively large quantities of blood, but instead of digesting it, apparently subsist mainly on the substances dissolved in the plasma. Owing to the nature of their habitat, it is not necessary for the digestive function of the helminths to be very highly developed. Even the free-living stages of the hookworm, which are able to subsist only on living bacteria, apparently depend for nourishment upon the vital functions of another organism.

Growth. The factors which determine the initiation of growth of helminth parasites in certain hosts and not in others are not at all understood. There are apparently two conflicting sets of evidence. On the one hand, it can be shown that worms are not sensitive to wide variations in certain factors in their environment, such as hydrogen-ion concentration, oxygen tension, temperature, etc. On the other hand, there is the frequent rigid restriction of parasites to certain species of hosts. Since it is not possible at present to cultivate any of the parasitic stages of the helminths of vertebrates in artificial media, it is difficult to determine the essential factors which govern the development of the worms.

The experiments of Heller (1933), which showed that *Trichinella spiralis* larvae were unable to develop when enclosed in semi-permeable collodion sacs suspended in the intestines of rats, have already been mentioned. Further evidence concerning the factors influencing the development of this parasite is afforded by some recent experiments by the author (McCoy, 1934). Sterile trichina larvae were introduced into the amniotic sac of rat embryos, and a certain proportion were able to develop normally to the adult stage. These experiments, at least, show that certain of the factors such as digestive enzymes, bacteria or their products, which are present in their normal intestinal environment, are not necessary for the development of the worms. It may be pointed out that *Trichinella spiralis* is noted for its ability to develop in a wide variety of different hosts.

Scott (1928) reported an interesting observation regarding the development of hookworm larvae in dogs. When an animal was given

a number of larvae by mouth, only a certain percentage of them developed in the intestine. If the dog was killed several weeks after infection, undeveloped larvae could be recovered from the intestine along with mature worms. These larvae were not incapable of development, for, when fed to a second dog, approximately the same percentage of them developed as would be expected if fresh larvae had been used. From these observations, it appears that in the case of the dog hookworm, even such individual variations as occur in the intestines of different host animals of the same species may have a significant effect on the ability of the parasite to develop.

Information concerning the rate of growth of helminth parasites is most complete in the case of the dog hookworm, *Ancylostoma caninum*. Scott (1929) found that measurements of the length of developing adult stages of the dog hookworm were fitted reasonably well by a logistic curve. In other words, their growth curve is similar to that of other types of organisms. The growth in length of the first two larval (free-living) stages of the dog hookworm may also be fitted by logistic curves (McCoy, 1930). It is interesting to note that the rate of growth of the adult stage, as shown by the *b* constant of Scott's curve, is approximately the same as that shown by the two larval stages when cultured at 24.5°C., a temperature which may be considered normal for dog hookworm larvae. In other words, different stages in the life cycle of the parasite show the same intrinsic rate of growth under normal conditions, even though the environments are radically different. The temperature of 37.5°C. in the dog's intestine, which is normal for the development of adult worms, is 13° higher than the temperature at which the larvae developed at the same intrinsic rate as did the adult worms.

Reproduction. One of the most pronounced effects of the parasitic mode of life on helminths is the enormous development of their reproductive capacities. In order to compensate for the numerous hazards encountered in completing their complicated life cycles, extremely large numbers of eggs are produced. For instance, a female *Ascaris lumbricoides*, according to Cram (1925) may contain at one time as many as 27,000,000 eggs, and regularly produces at least 200,000 eggs per day (Brown and Cort, 1927). The egg production is much less for the smaller helminths but is still a spectacular figure compared to that of free-living worms. The following are some examples of the number of eggs produced per day: the new-world hookworm, *Necator americanus*, 9,000 (Stoll, 1923); the old-world hookworm, *Ancylostoma duodenale*,

24,000 (Augustine *et al.*, 1928); the human intestinal fluke, *Fasciolopsis buski*, 25,000 (Stoll, Cort and Kwei, 1927).

In addition to producing large numbers of eggs, the trematodes and cestodes possess certain other devices which enhance the chances of completing their life cycles. Hermaphroditism and self-fertilization, which is the rule among these two groups, eliminates the hazard of cross-fertilization. Also, the larval stages of many species possess the power of extensive asexual reproduction in their intermediate hosts. As many as 10,000 cercariae may result from the asexual reproduction of a single miracidium in its snail intermediate host. Among the cestodes, the germinal epithelium of an hydatid cyst (*Echinococcus granulosus*) may produce hundreds of thousands of scolices, all the result of the development from a single larva.

The reproductive organs comprise a large proportion of the body weight of most helminths, and the process of producing such large numbers of eggs probably utilizes the major portion of the total energy requirements of the worms. The study of the metabolic processes involved in building up the protoplasm and yolk material necessary for the eggs presents many experimental difficulties and has not been attempted. Consequently, little information is available concerning the physiological mechanism of reproduction. Recent work on immunity reactions of the host against helminth parasites has demonstrated that the reproductive power of worms surviving in "immune" animals may be greatly inhibited. Female dog hookworms (*Ancylostoma caninum*), which normally produce 16,000 eggs per day, on the average produce only one-third this figure when present in dogs whose resistance has been increased by repeated infection (McCoy, 1931). An even more pronounced inhibition of the egg production of female worms persisting in partially immune rats has been reported by Chandler (1932) in the case of the rat nematode, *Nippostrongylus muris*. The factors responsible for this decreased egg production have not as yet been elucidated.

Secretions. 1. *Anti-enzymes.* The question has often arisen as to just what mechanism protects intestinal parasites from digestion within their host. It is well known that worms which have died inside the host may sometimes be observed partially digested in the feces. Occasionally, particularly in the case of nematodes, the cuticula of the worm may remain practically intact, even after most of the other tissues of the worm have disappeared. This fact suggests that the resistant

nature of the cuticula of helminths affords their first and probably most important protection against the digestive juices of their host.

Weinland (1903) reported evidence that intestinal helminths may also be protected from digestion through the elaboration of specific anti-enzymes. Using more exact methods, Hamill (1906) definitely demonstrated the presence of an anti-tryptic enzyme in water extracts of ascaris. This anti-enzyme withstood boiling for 5 minutes, providing the extract was acid or neutral; if alkaline, it was quickly destroyed. Fetterolf (1907) contributed another report demonstrating the existence of anti-enzymes in the human beef tapeworm, *Taenia saginata*.

Harned and Nash (1932) have recently restudied the anti-enzymes of ascaris. They found that the water extract of the worms contained not only an anti-trypsin, but also an anti-pepsin and a protease, active in alkaline solution, which was not inhibited by the anti-trypsin. The anti-trypsin could be separated from the other enzymes present in the extract by fractional precipitation with alcohol. In this way, it was possible to prepare highly concentrated extracts of the anti-trypsin.

Stewart and Shearer (1933) studied an anti-peptic enzyme present in nematode parasites of sheep, and suggested "nezyme" as a term for anti-enzymes. They believed that protein digestion in the sheep was lessened in the presence of large numbers of parasites, and attributed this effect to the elaboration of the anti-enzyme.

The observations cited above suggest that anti-enzymes may play an active part in protecting intestinal worms from digestion by their host, at least as long as the organisms are alive. The resistant cuticula probably serves to protect the main body of the worm, while the anti-enzymes protect the internal tissues from the host's digestive juices which may enter the various openings in the body surface. There is also the suggestion that in the presence of large numbers of worms, anti-enzymes may retard the digestive processes of the host.

2. *Anti-coagulins.* Loeb and Smith (1904) first demonstrated the presence of anti-coagulins in nematodes. They found that saline extracts of the dog hookworm, *Ancylostoma caninum*, inhibited clotting of dog's blood *in vitro* for periods occasionally as long as 24 hours. The active substance was present in the anterior half of the worms but not in the posterior half. It was very resistant to heat, being weakened by 15 minutes of boiling but not completely destroyed.

Schwartz (1921a) tested a number of different species of nematodes for the presence of an anti-coagulin, and found it present in some species but not in others. He concluded that the substance, when present, was

distinct from hemolysins, and was related physiologically to hirudin and snake venom. The worms which showed the anti-coagulin to a marked degree were zoologically related, mostly belonging to the family Strongylidae. Members of this group possess a buccal capsule adapted to lacerating the intestinal mucosa and are mostly blood-suckers.

Hoeppli and Feng (1933) have recently studied the inhibitory effect on the coagulation of rabbit blood by saline extracts of the anterior portion of the sheep hookworm, *Bunostomum trigonocephalum*. They found that the anti-coagulin was contained chiefly in extracts of the esophagus of the worms, while extracts of the cephalic glands showed only a slight inhibitory effect.

3. *Hemolysins.* Various investigators have found hemolysins in extracts of a number of different helminths. These hemolysins usually are weak, act slowly *in vitro*, are easily destroyed by heat, are non-specific in their action, and usually are demonstrable only in concentrated extracts (see review by Schwartz, 1921b). The hemolytic substances extracted are apparently closely bound to the tissues of the parasites, and no definite evidence has been reported that they are actually secreted by the worms.

Although it is possible that the hemolysins of worms may be of functional use in digestion in parasites that ingest blood, it is not likely that they are potent enough or are absorbed in large enough quantities to have an appreciable effect on the host. In spite of certain differences, most worm hemolysins probably belong in the same general category as tissue hemolysins.

4. *Histolytic ferments.* The liquefaction of host tissue by adult worms, resulting in a process of extra-intestinal digestion, has already been mentioned under the section on Nutrition. In addition to this, various investigators have reported observations which indicate that helminths may secrete histolytic ferments which aid them in migrating through host tissue. Larval stages of certain trematodes, for instance, possess definite glands, the apparent function of which is to produce this histolytic secretion (Faust, 1920). After the larvae have succeeded in penetrating their host, these glands disappear. The chemical nature of the histolytic ferments produced by helminths has not been studied.

5. *Toxins.* Many investigators have prepared extracts of worms which, when injected into animals, affect such functions as respiration and blood-pressure, and sometimes have produced symptoms of general intoxication and even death. The fact that more or less specific antibodies are developed by the host during helminthic infections has been

used as an argument to support the view that worm "toxins" may be absorbed by the host. However, definite evidence that worms produce an exotoxin such as is known for diphtheria and tetanus bacilli has not been reported. It is possible that under certain circumstances, such as the death and disintegration of the worm, the "toxins," more properly designated as endotoxins (see Taliaferro, 1929), may have appreciable effects on the host. This is particularly true of the worms which localize in such places as the lymph vessels (*Filaria bancrofti*) and bile passages (liver flukes). Under ordinary circumstances, it seems doubtful that helminth endotoxins are of very great significance in injuring the host.

Behavior reactions. It has already been mentioned that helminths possess a definitely developed nervous system, but very little is known of how it functions in the adult parasitic stages. Practically all of the work that has been done on the behavior of helminths has concerned the free-living stages. This is true for several reasons. 1. The larval stages are in general more easily obtained and kept alive than are the adult worms. 2. Many larval forms possess sense organs such as eye-spots, structures which are not found in the parasitic stages and consequently they present greater possibilities for behavior studies. 3. The behavior reactions of the infective larval stage of many parasites, for example, hookworm, are of practical importance in the infection of the host.

It is, of course, known that adult helminths are able to react to touch stimuli, and to heat and cold. In addition, there is considerable indirect evidence from the migration and localization of the worms that they react to chemical stimuli. For instance, young stages of the human liver fluke, *Clonorchis sinensis*, after being freed from their cysts in the duodenum, show a remarkable ability to find the small opening of the common bile duct in order to migrate to the site of their development. Another example, indicating reaction to chemical stimuli, is the constancy with which female worms are fertilized by the males even when small numbers of parasites are present. Doerr and Menzi (1933) found that the feeding of rats with only two trichina larvae was usually sufficient to establish infection of the muscles. In other words, a single male worm in the space of the rat's intestine must have been able to find and fertilize the single female worm present, an individual only 2 or 3 mm. in length.

Turning to the consideration of the behavior of the larval stages of helminths, a number of reports of their ability to react to chemical

stimuli have been recorded. The miracidium, a stage in the life cycle of trematodes, is apparently definitely attracted by the mucous secretion of its snail intermediate host (Faust and Meleney, 1924; Barlow, 1925, etc.). The attraction takes place whenever the miracidium comes within a range of a few millimeters of the snail, and the reaction is apparently specific in that the secretions of species of snails that are unsuitable hosts do not attract the miracidium.

On the other hand, cercariae, another stage in the life cycle of trematodes, usually do not exhibit chemotactic reactions to their hosts, but show phototropic responses which may have practical significance in their life history. For instance, *Cercaria floridensis*, a species which penetrates and encysts in small fish, swims upward when stimulated by a shadow. Miller and McCoy (1930) found that larger numbers of cercariae succeeded in penetrating the fish when the fish were confined to the upper levels of an aquarium and exposed to cercariae in sunlight, than when they were exposed under the same conditions in total darkness.

The responses of trematode cercariae to changes of light intensity have also been widely studied as a problem in general physiology (Miller, 1926, and others). In general, the mechanism of these responses appears to be similar to that shown by many other invertebrates. Bevelander (1933) working with the cercaria of *Bucephalus elegans*, a trematode parasite of fish, found that the change of light intensity must be fairly rapid in order to induce a response, and concluded, therefore, that it was of the nature of a shock reaction. He also reported that the reaction time of the cercariae varied inversely with both temperature and intensity of stimulus. Miller and Mahaffey (1930) reported evidence that a dual mechanism must be involved in the response of *Cercaria hamata* to shadow and to touch stimuli. The cercariae could be kept in almost continuous locomotion by repeated touch stimuli, whereas a relatively long interval had to intervene between shadow stimuli in order to obtain a regular response.

The behavior of certain nematode larvae which penetrate the skin of their host (notably Strongyloides and hookworm) has been extensively investigated in an attempt to explain the activity of the larvae during their free-living existence (Payne, 1923; Fülleborn, 1924, 1932). At first there was a tendency to ascribe the behavior or the larvae to numerous tropisms, mechanical responses to such stimuli as touch, heat, light, water, chemicals, gravity, water currents, osmotic pressure, etc. The evidence regarding the existence of these tropisms is conflicting, chiefly

because it is difficult experimentally to separate the influences of the various factors.

Most investigators agree that hookworm larvae exhibit positive thermotropism, that is, are attracted by heat (Khalil, 1922; Fülleborn, 1924). But Lane (1930) explains this response as being due merely to the increased activity of the larvae at the higher temperature. Recently, Wakeshima (1933a) has claimed that hookworm larvae are positively attracted by tissue extracts, and also by blood and bile, but are repelled by certain strong chemicals. These results do not agree with other investigators who, in general, have been unable to demonstrate chemotropisms. Wakeshima's claim (1933b) that hookworm larvae show a positive phototropism, also is not in agreement with earlier work. Lane (1930) came to the conclusion that there is no adequate evidence for the existence in hookworm larvae of any tropisms other than thigmotropism, or response to contact stimuli, and believed that this response together with the increased activity of the larvae at body temperature is sufficient to explain the ability of the larvae to penetrate the skin of their host.

SUMMARY

The effect of the parasitic mode of life on the morphology and life cycle of the helminth parasites is well known and discussed in most text-books, but the effect on their physiology is not often mentioned, chiefly because little definite information has been available. The work, summarized in the foregoing review, indicates that the chief influence of parasitic existence on the physiological processes of helminths has concerned their secretions, reproduction and metabolism. The elaboration of anti-enzymes, anti-coagulins, and histolytic ferments may definitely be considered as adaptations to meet the exigencies of the environment inside the host. The enormous development of the reproductive capacities of helminths represents a compensation for the numerous hazards encountered in completing their complicated life cycles. Also, apparently helminths have become adapted to conditions of very low oxygen tension by developing an anaerobic type of metabolism in which glycogen is split into carbon dioxide and fatty acids. Compared with the metabolism of higher animals, this method of gaining energy is a very inefficient one, but is apparently adequate to meet the requirements of the environment. The free-living stages of helminth parasites require oxygen for their development, and apparently retain an aerobic type of metabolism. It is interesting that different stages in the life cycle of the same organism should show such wide differences in their physiological processes.

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RECENT WORK ON CARBON DIOXIDE TRANSPORT BY THE BLOOD

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When blood is drawn from an animal under normal physiological conditions, and is treated with excess of weak acid (e.g., lactic or tartaric acid) it is found to give up 40 to 60 cc. of carbon dioxide per 100 cc. of blood when submitted to a vacuum: it is thus said to contain a total CO_2 content of 40 to 60 vols. per cent CO_2. Of this a small part is in simple physical solution as dissolved CO_2, whilst the remainder, which is often called the bound CO_2, is in one or more forms of chemical combination.

During the past fifteen years, the state of carbon dioxide in the blood and its mode of transport in the circulation has attracted much attention amongst physiologists, especially in America, Denmark and Great Britain. By 1928 it had been almost universally agreed that dissolved carbon dioxide [CO_2], carbonic acid [H_2CO_3] and bicarbonate ions [$H\overline{CO}_3$] constituted for practical purposes the only three forms in which carbon dioxide is carried in the blood. The evidence for this view together with the description of the changes in distribution between the three forms which occur during the respiratory cycle has been most lucidly presented by van Slyke (1922), Henderson (1928) and Peters and van Slyke (1931). It is not the purpose of the present review to recover ground which has already been covered so well; the object is rather to consider how far the picture which seemed firmly framed in 1928 requires re-setting in light of the interesting and unexpected developments which have taken place since that date. The "1928" viewpoint is indeed so well known as only to need the following very brief summary.

When CO_2 is taken up by a watery fluid the following reactions occur

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons \overset{+}{H} + H\overline{CO}_3$$

The equilibrium constants of these several processes are such that in plain water the system only proceeds very slightly to the right: in blood,

however, the buffers present remove the $\overset{+}{H}$ ions so effectively that the resultant displacement of the equilibria to the right is considered to lead to 95 per cent (roughly) of the CO_2 taken up being in the form of $H\overline{CO_3}$ ions. The main buffer responsible for this happening is the alkali salt of hemoglobin which is often written B Hb (mostly potassium hemoglobinate) present in the red cells. The hemoglobin ions $\overline{Hb}$ formed by the ionisation of this salt combine with hydrogen ions to form unionised (or less fully ionised) hemoglobin molecules HHb. To a lesser extent the salts of the plasma proteins, and the $\overline{\overline{HPO_4}}$ ions of the plasma act in the same way.

The ability of the alkali hemoglobinate to remove $\overset{+}{H}$ ions is enhanced by the fact that simultaneously with the entrance into the blood of CO_2, the hemoglobin loses O_2. Now reduced hemoglobin is known to be a "weaker" acid than oxyhemoglobin; this is probably because there is one particular hydrogen ion (named by van Slyke the oxy-labile hydrogen ion) which combines with, or dissociates from, a spot close to each hematin nucleus in the hemoglobin molecule: attachment of oxygen to the hematin nucleus increases greatly the degree of dissociation of this $\overset{+}{H}$ ion, [i.e., about 20 times as judged by its effect on the ionisation constant] whereas removal of oxygen conversely increases the ease of binding of $\overset{+}{H}$ ions at this point. Whatever the cause, there is no doubt of the observed fact, and on this account reduced hemoglobin at a given pH "mops" up free $\overset{+}{H}$ ions from the surrounding solution more readily than oxyhemoglobin does. The reduction of the hemoglobin thus ipso facto enables the blood to bind more CO_2 as bicarbonate: 50 per cent or more of the CO_2 carrying power of blood under physiological conditions is attributed to this factor.

During the uptake of CO_2 by the blood, there is a tendency for the $H\overline{CO_3}$ concentration in the corpuscle to increase more markedly than in the plasma, owing to the preponderance of buffer salts in the red cells. The Donnan equilibrium between the red cells and the plasma is thereby upset, and some of the $H\overline{CO_3}$ ions from inside the red cells exchange for $\overline{Cl}$ ions from the plasma until equilibrium is once more restored. The result of this interchange ("chloride shift") is that the superior buffering power and CO_2 carrying of the red cells are shared out with the plasma, which is, per se, relatively inferior in these respects. The capacity of the plasma is thereby brought up to the level of or even beyond that of the red cells. On this account the process has often been called "sec-

ondary buffering." Owing to the osmotic changes involved in these processes a small amount of water also passes from the plasma to the red cells during CO_2 uptake.

When the blood gives out CO_2 in the lungs the above changes are all reversed. Figure 1 gives a schematic summary of the more important of these processes considered, for convenience, for the case of CO_2 output from the blood.

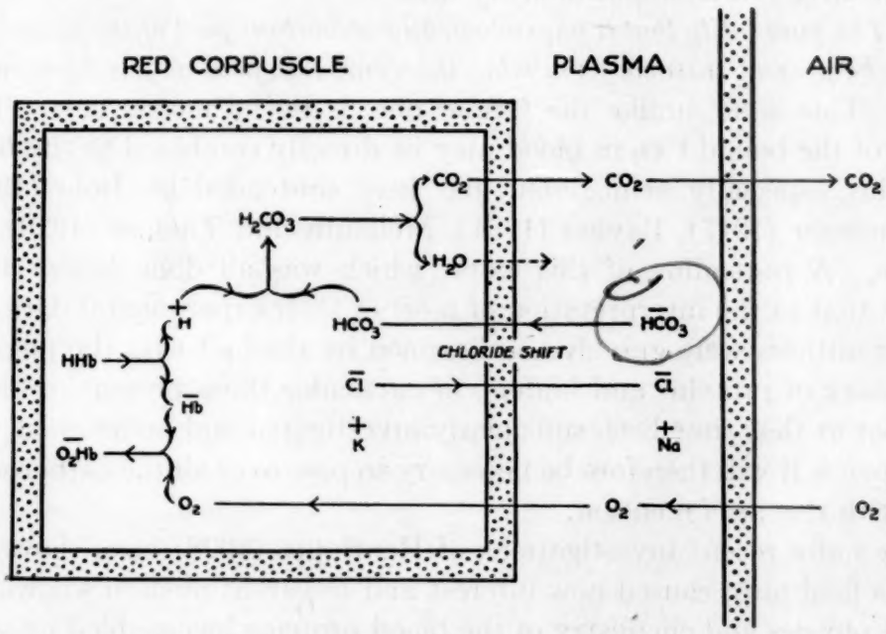

Fig. 1. Schematic summary of main processes which occur within the blood, during output of carbon dioxide and uptake of oxygen in the lung according to "1928 view-point."

During the past six years attention has been directed towards two main issues which did not figure at all in the "1928" scheme. These were as follows:

1. *The speed at which gaseous carbon dioxide could be formed from $H\overline{CO_3}$ ions under the conditions of pH and temperature existing in the blood capillaries in the lung.* The processes, summarised in figure 1, had all been discovered or deduced from experiments in which blood, or certain fractions thereof, was equilibrated in tonometers outside the body with gas mixtures containing various proportions of CO_2. Such equilibrations usually take 15 minutes or more to reach an end point: it was tacitly assumed that conclusions derived therefrom could be applied to events in the actual blood capillary, wherein, however, the blood when in active circulation, may spend a time not of 15 minutes but only of 1 second or so.

The first hint, in the literature known to the reviewer, that the dynamics as well as the equilibrium states of these processes might be worthy of attention, is that by Hartridge and Roughton (1925) who, following on their studies of the kinetics of the rapid chemical reaction between oxygen and hemoglobin in blood, pointed out the desirability of studying the kinetics of the carbon dioxide processes in blood likewise. It is, however, to the pioneer work of Henriques (1928) that the great stimulus in this direction is unquestionably due.

2. *The possibility that a physiologically important part of the bound CO_2 might be present in some form other than carbonic acid or free bicarbonate ions.* This issue, unlike the former one, is by no means new. That some of the bound CO_2 in blood may be directly combined to the blood proteins, especially hemoglobin, has been contended by Bohr (1909), Buckmaster (1917), Bayliss (1924), Mellanby and Thomas (1920) and others. A re-reading of this work, which was all done before 1925, shows that in the interpretation of most of their experimental data, the earlier authors were gravely handicapped by the fact that the physical chemistry of proteins and buffers, in particular those present in blood, had not at that time been sufficiently investigated and understood. In this review it will therefore be necessary to pass over all the earlier work just with this brief mention.

The more recent investigations of Henriques (1928) have, however, in this field also, caused new interest and research: modern knowledge of the physics and chemistry of the blood proteins has enabled present-day investigators to avoid some of the pitfalls, into which their predecessors fell, but there may well be other pitfalls of which present-day research is ignorant.

The plan of this review is as follows:

In section I: To recount and appraise the new knowledge which has been revealed since 1928 by the study of the kinetics of the carbon dioxide processes in blood.

In section II: To summarise the new evidence dealing with the possible existence of one fraction or more of the bound CO_2 of the blood in some form other than free bicarbonate ions; and lastly to present an amended edition of figure 1, in accordance with existing views.

SECTION I: THE KINETICS OF THE CARBON DIOXIDE PROCESSES IN BLOOD—CARBONIC ANHYDRASE. It will be useful to begin by stating what is known a priori as to the rate of the individual processes schematised in figure 1. Most of them clearly belong to the type of "simple ionic reactions," i.e., to reactions in which either a molecule dissociates into its con-

stituent ions, or the latter recombine to form the undissociated molecule. Now simple ionic reactions are generally thought by physical chemists to be practically instantaneous: that such ionic reactions do in fact proceed to within 1 per cent of their end-point in less than 1/1000 second has been proved experimentally by the researches of Hartridge and Roughton (1923–1926), Saal (1928a), Roughton (1930) and Lamer and Read (1930). None of these ionic processes therefore would be expected to limit the rate at which CO_2 could be taken up, or given out, by the blood.

There are, however, two processes in figure 1 which do not fall within this category. These are

a. The dehydration of carbonic acid to carbon dioxide and vice versa, i.e., $H_2CO_3 \rightleftharpoons H_2O + CO_2$.

b. The exchange of chloride ions for bicarbonate ions between the plasma and the corpuscle (and vice versa). Little is known at present as to the rate of *b* under physiological conditions, and the question will therefore only be treated shortly at the end of this section, the main attention of which will be given to *a*.

THE KINETICS OF THE REACTION $H_2CO_3 \rightleftharpoons H_2O + CO_2$ in absence of catalyst. The kinetics of the $H_2CO_3 \rightleftharpoons CO_2 + H_2O$ reaction in inorganic solutions have been studied by several authors beginning with Thiel (1913). It has been found that the processes concerned are relatively slow. The first work of a really comprehensive kind is that of Faurholt (1924): his results and views of the mechanism of the reaction have been confirmed over a very wide range of conditions by Saal (1928b) Brinkman, Margaria and Roughton (1933) and Stadie and O'Brien (1933), all of whom used different methods of observation.

According to Faurholt, the kinetics of the reaction at pH < 8.0 can be expressed by the equation

$$\frac{d[CO_2]}{dt} = k_{CO_2}[CO_2] + k_{H_2CO_3}[H_2CO_3] \qquad (1)$$

i.e., the equation given by the Law of Mass Action as applied to the reversible reaction

$$CO_2 + H_2O \rightleftharpoons H_2CO_3$$

At pH > 8.0 a second equation has also to be taken into account, viz.,

$$\frac{d[CO_2]}{dt} = k_{CO_2 \cdot OH}[CO_2][\overline{OH}] + k_{HCO_3}[H\overline{CO_3}] \qquad (2)$$

Between pH 8.0 and pH 10.0 both equations are important, but at pH > 10 the [$\overline{OH}$] concentration is large enough to make equation (2) the dominant one. Equation (2) can be obtained either by applying the Law of Mass Action to the reaction $CO_2 + \overline{OH} \rightleftharpoons H\overline{CO_3}$, or by supposing that the reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$ is catalysed by $O\bar{H}$ ions the concentration of which only becomes large enough to have appreciable catalytic effect when the pH is raised above 8.0.

There is at present no way of distinguishing between these two mechanisms: the pH range in which this equation operates is, however, more alkaline than that met with in the blood.

Brinkman, Buytendyk and Mook (1927) suggested that at pH <8.0, Faurholt's equation (1) was incorrect and ought to be replaced by the equation

$$\frac{d\,[CO_2]}{dt} = k'_{CO_2}\,[CO_2]\,[\overset{+}{H}] + k'_{H_2CO_3}[H_2CO_3]\,[\overset{+}{H}].$$

Brinkman, Margaria and Roughton (1933), however, showed later that the experiments on which this suggestion was based, were vitiated by a technical error which made them useless for purposes of calculation.

It may be useful to summarise here the various methods by which the kinetics of the reaction have been studied, since several of these are being applied now to the study of the reaction when catalysed by the enzyme, carbonic anhydrase (v. infra):

A. *Rate of dehydration, i.e., $H_2CO_3 \rightarrow H_2O + CO_2$ of carbonic acid.* By mixing a solution of bicarbonate with a suitable buffer solution and then following:

1. The amount of CO_2 evolved into the gas phase after various times by means of a manometer v. Brinkman, Margaria and Roughton (1933) pH range studied 5.6 – 7.0.

2. The change in concentration of [CO_2] in the liquid phase with time.

(a) By Faurholt's Carbamino method (1924). After the reaction has proceeded for a desired time it is suddenly stopped by throwing in an excess of dimethylamine followed by $BaCl_2$. The whole of the H_2CO_3 and $H\overline{CO_3}$ are at once turned into $\overline{\overline{CO_3}}$ and precipitated as $BaCO_3$: the dissolved CO_2, however, combines almost completely with the dimethylamine to form the carbamino compound, and, since the latter is not precipitated by barium, it is easily separated from the $BaCO_3$ precipitate by centrifuging and then estimated, thus giving the [CO_2] in the reacting solution at time *t*. pH range studied 5.6 – 7.0.

(b) By Dirken and Mook's method (1930). The two reagents are

mixed together in a Hartridge-Roughton rapid reaction apparatus: the streaming fluid, after leaving the mixing chamber travels through a variable volume of tubing, and thence passes in the form of a fine jet through a small chamber containing gaseous CO_2. From the rate of absorption of the latter from the gas phase, the concentration of CO_2 in the moving jet is obtained. pH range studied 0.0 to 2.0.

3. The subsequent changes in pH. From these the corresponding changes in $[H\overline{CO}_3]$ and $[CO_2]$ can be readily calculated.

(a) The two reagents are mixed together by a rapid stirrer and the pH in the stationary fluid at subsequent instants is registered electrometrically. Brinkman, Buytendyke and Mook (1927) pH range studied 5.0 to 6.0. Stadie and O'Brien (1933).

(b) The two reagents are mixed together in a Hartridge-Roughton rapid reaction velocity apparatus (1923), and the pH in the streaming fluid at various instants after mixture determined by measurements (i) of electrical conductivity (Saal, 1928b) or (ii) by the hydrogen electrode (Saal, 1928b) or (iii) by indicator colour changes (Brinkman, Margaria and Roughton, 1933). pH range studied 3.0 to 5.0.

B. *The rate of hydration of carbon dioxide, i.e.,* $CO_2 + H_2O \rightarrow H_2CO_3$.
1. By shaking a suitable buffer mixture with gaseous CO_2, and following the CO_2 uptake manometrically (Brinkman, Margaria and Roughton, 1933).

2, 3. By mixing a solution of dissolved CO_2 with a suitable buffer solution and then using one of the methods listed under headings A(2) or A(3) for the dehydration reaction velocity. The pH range studied in all these cases was 6.0 to 8.0.

The results obtained by these various methods are collected in table 1, and are seen to be satisfactorily consistent, apart from the values for $k_{H_2CO_3}$ by method 2a (Faurholt) and by method 2b (Dirken and Mook's method). According to Saal (1928b) method 2a is subject to a possible error of $\pm$ 50 per cent, and so according to Brinkman, Margaria and Roughton (1933) was their result by method 2b. The most accurate results are probably those given by method 1. Considering the wide variety of methods, and conditions (the time for half reaction ranged from 0.05 second to 250 seconds), the equation

$$\frac{d[CO_2]}{dt} = -k_{CO_2}[CO_2] + k_{H_2CO_3}[H_2CO_3]$$

is well supported by the experimental evidence.

A few more words should be added about the kinetics of the dehydration velocity. Since the ionisation of carbonic acid is practically instantaneous, we may, throughout the process (neglecting for the moment activity coefficients) put

$$K_{H_2CO_3}[H_2CO_3] = [\overset{+}{H}] \times [H\overline{CO}_3] \quad (3)$$

where $K_{H_2CO_3}$ is the first ionisation constant of H_2CO_3.

TABLE 1

A. Values of dehydration velocity constant $k_{H_2CO_3}$ in mols./sec.

TEMPERATURE	METHOD						
	(1)	(2a)	(2b)	(3a)	($3b_1$)	($3b_2$)	($3b_3$)
pH range studied	5.6–7.0	5.6–7.0	0–2.0	5.0–6.0	3.0–5.0	3.0–5.0	3.0–5.0
0°C.	1.7*	2.7*†		1.4			
18°C.	10.4*	16.3*†	(17.0)		12.4	10.3	11.8
38°C.	70‡ by extrapolation using the Arrhenius equation)						

B. Values of hydration velocity constant k_{CO_2} in mols./sec.

TEMPERATURE	METHOD			
	(1)	(2a)	(3a)	($3b_3$)
pH range studied	6.0–8.0	6.0–8.0	6.0–8.0	6.0–8.0
0°C.	0.0026	0.0030†	0.0027	
18°C.	0.021	0.025†		0.026
38°C.	0.161 (by extrapolation)			

* Assuming that $K_{H_2CO_3}$ at 0°C. = $K_{H_2CO_3}$ at 18°C. = 2×10^{-4}.

† The values of the velocity constants given in Faurholt's (1924) paper have to be multiplied by 2.3 to bring them to the usual units of mols./sec. As Stadie and O'Brien (1933) point out, Brinkman, Margaria and Roughton (1933) failed to notice this, and hence concluded that there was a discrepancy between their results and those of Faurholt, both as regards $k_{H_2CO_3}$ and k_{CO_2}, instead of for $k_{H_2CO_3}$ only as in table 1.

‡ The extrapolated value of 140, given in table 1 of Stadie and O'Brien's 1933 paper appears to be a miscalculation.

Therefore in the early stages when the rate of the back reaction is negligible

$$d/dt\,[CO_2] = k_{H_2CO_3}\,[H_2CO_3]$$

$$= k_{H_2CO_3} \frac{[\overset{+}{H}]\,[H\overline{CO}_3]}{K_{H_2CO_3}} \quad (4)$$

The value of K_{HCO_3} is of the order of 2×10^{-4} at room temperature but is not exactly known, nor is its variation with temperature known. At and above pH 5 it follows from equation (3) that H_2CO_3 is proportional to, but not more than 5 per cent of the $[H\overline{CO}_3]$, so that in these circumstances the initial rate of the reaction is proportional to the initial $[H\overline{CO}_3]$ and to the $[\overset{+}{H}]$. As the pH is made more acid, the initial rate, at constant initial $[H\overline{CO}_3]$ increases to a maximum which it reaches at pH 2 about, for at this pH almost the whole of the $H\overline{CO}_3$ at once combines with $\overset{+}{H}$ ions to form H_2CO_3, the active mass of which is not appreciably increased by further acidification. At this pH the value of $k_{H_2CO_3}$ can therefore be calculated without knowledge of $K_{H_2CO_3}$. In the physiological range of pH (viz., 6.0 – 8.0), however, experiments, such as those by method 1 (table 1), on the rate of reaction only enable the fraction $k_{H_2CO_3}/K_{H_2CO_3}$ to be directly calculated. The absolute values of $k_{H_2CO_3}$ given in table 1 at pH > 5.0, have all been obtained by multiplying the calculated values of $k_{H_2CO_3}/K_{H_2CO_3}$ by 2.0×10^{-4} and may therefore need correction when accurate values for $K_{H_2CO_3}$ are available. This does not, however, affect the calculation of the speed of dehydration processes in the physiological range of pH, since it is the fraction $k_{H_2CO_3}/K_{H_2CO_3}$, rather than the simple velocity constant $k_{H_2CO_3}$, that always appears in the equations. This fraction can be *accurately* obtained from the column headed method 1 in table 1, by simply dividing the figure given there by 2×10^{-4}, and thus no independent knowledge of the value of $K_{H_2CO_3}$ is necessary for such physiological calculations.

Equation (3) should properly be written

$$K_{H_2CO_3}[H_2CO_3] = a_H \times f_{HCO_3}[H\overline{CO}_3]$$

where a_H is the hydrogen ion activity of the solution (as given by electrometric measurements) and f_{HCO_3} is the activity coefficient of the bicarbonate ion.

Equation (4) should thus be replaced by

$$d/dt\,[CO_2] = k_{H_2CO_3} \frac{a_H f_{HCO_3}[H\overline{CO}_3]}{K_{H_2CO_3}} \qquad (5)$$

The value of f_{HCO_3} in blood is about 0.6 (Van Slyke, Hastings, Murray and Sendroy, 1925) so that in calculations relative to conditions in blood activity corrections cannot be left out of account.

THE RATE OF FORMATION OF CO_2 FROM BICARBONATE IN THE BLOOD, IF NO CATALYST FOR THE REACTION $H_2CO_3 \rightarrow CO_2 + H_2O$ BE PRESENT. Henriques (1928) was the first to publish calculations as to the proportion of the CO_2 evolved from the blood during its passage through the lung, which could be formed from bicarbonate under the available conditions of pH, temperature and time, if no catalyst was present.

Assuming (i) the pH of the blood in the lung capillaries = 7.4
(ii) temperature = 37°C.
(iii) the value of $k_{H_2CO_3}$ extrapolated from Faurholt's data at 0°C. and 18°C.
(iv) the average time spent by the blood in the lung capillaries = 1 second.
(v) that the rate at which the reaction proceeds towards equilibrium conforms to the equation used by Faurholt in computing tables 24, 25 and 26 of his 1924 paper.

Henriques calculated that 16.6 per cent of the evolved CO_2 could be furnished by the reaction $H_2CO_3 \rightarrow CO_2 + H_2O$ without the aid of any catalyst.

Assumption (v), however, does not hold good for the actual conditions in the lung, and leads to too high results for two reasons:

(α) Faurholt's equation gives the rate at which a bicarbonate solution, containing initially no dissolved CO_2 proceeds towards equilibrium in the all-liquid phase. At the beginning of such a process, $[CO_2]$ being zero there is no back reaction. In the lung capillary, however, the $[CO_2]$ cannot even in the blood as it enters, be less than the value which is in equilibrium with the CO_2 pressure in the alveolar air, assuming, as is generally agreed, that CO_2 only diffuses through the lung membrane and is not actively excreted. Even at the beginning of the capillary then the rate of the back reaction, i.e., $k_{CO_2}[CO_2]$, must be very appreciable.

(β) In the conditions corresponding to Faurholt's equation it can be shown that at pH 7.4, equilibrium would be reached when 5 per cent of the initial $H\overline{CO_3}$ concentration had been converted into CO_2, and that it is 16.6 per cent of this 5 per cent which could be converted into CO_2 in one second. Actually, however, when the blood passes through the lungs 7 to 20 per cent of the initial $H\overline{CO_3}$ in the venous blood dissociates into CO_2, according to whether the body is at rest or at work, and thus the proportion of the evolved CO_2, which could be formed in 1 second by the reaction $H_2CO_3 \rightleftharpoons H_2O + CO_2$ in absence of catalyst, is not 16.6 per cent, but in the case of rest $16.6 \times 5/7$, i.e., 12 per cent, and in the case of work $16.6 \times 5/20$, i.e., 4.1 per cent.

The problem has therefore been worked out again by Roughton (1934) who shows that the percentage of evolved CO_2, x, which could be furnished by the reaction $H_2CO_3 \rightarrow CO_2 + H_2O$ without the aid of any catalyst is given roughly by the equation

$$\mathrm{Log_e}\left(\frac{100}{100 - x}\right) = \frac{k_{H_2CO_3}}{K_{H_2CO_3}} f_{HCO_3} a_H \qquad (6)$$

Assuming at 38°C.,

$$\frac{k_{H_2CO_3}}{K_{H_2CO_3}} = \frac{70}{2 \times 10^{-4}} = 3.5 \times 10^5$$

$$f_{H_2CO_3} = 0.6$$

The results obtained for the blood of A.V.B. the data on which are quoted by Henderson (1928, pp. 195, 243), are shown in table 2A.

These figures give a much less favourable picture of the efficacy of the $H_2CO_3 \rightleftharpoons CO_2 + H_2O$ reaction, in absence of catalyst, than does the original calculation of Henriques. From table 2A it appears that not more than 2 per cent, at most, of the CO_2 evolved in the lung, could be furnished by the uncatalysed, $H_2CO_3 \rightleftharpoons CO_2 + H_2O$, reaction within one second.

It is generally thought that the blood in the lung capillaries comes practically into equilibrium with the CO_2 of the alveolar air. Equation (6) has also been used to calculate how long it would take the reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$ to reach to within 10 per cent of equilibrium under conditions obtaining in the lung. The figures are given in table 2B, and are more than a hundred-fold greater than the average time assumed to be spent by the blood in traversing the lung capillary. If the near approach to equilibrium, which is thought actually to occur, is to be brought about by the reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$, the blood must contain some catalyst which accelerates it under physiological conditions at least 150 times.[1]

[1] Less catalytic acceleration would be needed if the average time of the blood in the lung capillaries was more than one second. Any precise estimate of the average time is made very difficult by the variability, both in the number of patent lung capillaries and in the character of blood flow therein (see especially the recent work of Wearn et al., 1934). The average time may well be much more than one second, but it is unlikely that the minimal time is much longer. To be on the safe side there should be enough catalyst to cope with conditions in the fastest red cell to traverse the lung capillary.

As regards CO_2 uptake by the blood in the tissues it need only be said that calculations of the same type give exactly similar indications to those already found in the case of CO_2 output.

THE ENZYME CARBONIC ANHYDRASE. *Historical introduction.* After having found by calculation that the reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$ is not, in absence of catalyst, nearly fast enough to furnish CO_2 at the rate at which it is observed to escape in the expired air, Henriques' (1928) next step was to determine the actual speed at which CO_2 escapes from blood, in vitro, when shaken violently in a vacuum. He noted that, whereas the rate of escape from serum was of the order to be expected

TABLE 2

A. x = proportion of CO_2 evolved in the lung which could be furnished by the $H_2CO_3 \rightleftharpoons CO_2 + H_2O$ reaction in absence of catalyst in one second

CONDITION OF SUBJECT	SERUM		INTERIOR OF RED CELLS	
	α_H*	x	α_H*	x
		per cent		*per cent*
Rest	3.61×10^{-8}	0.8	7.69×10^{-8}	1.6
Work	4.84×10^{-8}	1.3	9.03×10^{-8}	1.9

* Average of arterial and venous values.

B. Time necessary for formation of 90 per cent of the CO_2 which is evolved in the lung via the reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$ in absence of catalyst

CONDITION OF SUBJECT	SERUM	INTERIOR OF RED CELLS
Rest	304 seconds	142 seconds
Work	227 seconds	121 seconds

from the pH and the velocity constants of the reaction, the rate of escape from hemoglobin solutions, especially in the early stages, was very much faster. This most important observation was undoubtedly the stimulus which has attracted other workers to the subject of this review during the past six years. To explain his finding, Henriques put forward two possible theories: *a*, that there was a catalyst for the reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$ present in the red cells; *b*, that the phenomenon was due to a rapid reversible reaction between hemoglobin and CO_2 of a direct type, analogous to the oxygen-hemoglobin reaction. He at first thought that the catalytic explanation was not feasible: this was quite justifiable in view of the data at his disposal at the time. The

work, by others, which followed shortly afterwards, showed, however, beyond doubt that both explanations are simultaneously true. In this section we shall deal only with the catalytic aspect. It will be convenient to postpone further consideration of Henriques' original results till the next section on the state of CO_2 in blood.

The first confirmation of Henriques' observation, that in hemoglobin solutions the rate of CO_2 evolution is greatly accelerated, was given by the work of Hawkins and Van Slyke (1930). These authors found that the acceleration persisted in blood solutions diluted 20-fold, and hence inclined strongly to the catalytic view of the phenomenon. Decisive evidence for the latter was brought forward in 1931 by Brinkman and Margaria (1931) [see also Dirken and Mook (1931)] who showed that blood or hemoglobin, even when diluted 1 in 20,000, still accelerated markedly the rate of evolution of CO_2 from mixtures of bicarbonate with buffer solutions of physiological pH. Since dialysed hemoglobin (purified by Adair's method), showed strong activity in high dilution, these authors attributed the catalytic power to hemoglobin itself.

The next investigation, by Brinkman, Margaria, Meldrum and Roughton (1932) showed that solutions of mammalian oxyhemoglobin, reduced hemoglobin and CO hemoglobin were all equally active as catalysts, but that on splitting the hemoglobin into hematin and globin, no activity was found in the hemin fraction, but on the other hand the globin fraction showed practically as much activity as the original hemoglobin from which it was prepared. These authors also found that although the catalyst is nearly always present in solutions of hemoglobin, the ratio hemoglobin/catalytic action varies very greatly for blood of different animals. In fish blood or frog blood the catalysis for a given amount of hemoglobin is much less than in mammalian blood, whilst in the hemoglobin containing blood of Planorbis and of the earthworm the catalytic action was practically absent. These findings suggested strongly that the catalyst was not hemoglobin itself, but was probably some substance which is often closely associated with the latter. Early in 1932 Meldrum and Roughton (1932 a,b,c) therefore set out to separate, if possible, the catalytic factor from hemoglobin.

In this they were speedily successful. The final of three methods, which they adopted, resembled in its initial stage Tsuchihashi's (1923) method of preparing catalase from blood. The details given by Meldrum and Roughton (1933a) are as follows:

Isolation of carbonic anhydrase. To 10 cc. washed ox red cells in a centrifuge tube are added 6 cc. H_2O and 4 cc. ethyl alcohol. The mix-

ture is then shaken at room temperature for about a minute with 5 cc. $CHCl_3$ at the end of which coagulation of the hemoglobin should be practically complete.

On centrifuging for about 10 minutes at 3500 r.p.m. a three-phasic system is formed, consisting of a top layer of solution, a central layer of denatured protein, and a bottom layer of chloroform.

The solution contains traces of hemoglobin of the order of 1 in 5000 in good preparations and much catalase. The yield is on the average about 50 per cent of the maximum; in different cases there were considerable variations from this figure both in upward and downward directions. The cause of this is not known but may be in part explained by varying amounts of catalyst adhering to the hemoglobin coagulum. Rubbing up the latter with water gave a solution of high activity on the one occasion when the test was made. On evaporating the catalyst solution to dryness in a vacuum desiccator a brown solid catalyst preparation is obtained containing methemoglobin and hemochromogen compounds. The yield is about 1 gram from 100 cc. of corpuscles. The activity is about 100 times greater than that of the whole blood and about 20 times greater than that of the total solids of the red cells.

The catalyst is extremely stable in this form and can be kept for many months even if exposed to the air. The brown solid readily dissolves in water or salt solutions to yield a clear solution, having the properties of the original (unevaporated) solution. In aqueous solution the catalyst keeps for many weeks in the ice chest. The rate of destruction varies with different samples, but in general the catalyst loses < 20 per cent activity in 5 to 6 weeks.

This crude solution of catalyst thus prepared is used as the basis for further purification. The dialysable impurities, such as the salts of the blood, were removed by ultrafiltration, with the aid of $4\frac{1}{2}$ per cent and 6 per cent. Bechold ultrafiltration membranes (purchased from Schleicher and Schüll).

The non-dialysable impurities proved more difficult to remove. Fractional precipitation was not successful, nor was adsorption, except in the case of two particular adsorbents, viz., 1, Willstätter's C-γ alumina cream, and 2, a suspension of roughly 1 per cent $Ca_3(PO)_2$. These adsorbents are both effective in removing selectively the other non-dialysable impurities, especially the traces of colouring (hematin) matter, without at the same time taking up serious amounts of carbonic anhydrase. The extent of the treatment was adjusted for each sample,

the mixture being filtered after each treatment with adsorbent. Finally on evaporating a colourless transparent solid was obtained.

The purest preparation made by this method showed an activity about 2000 times greater than that of the original whole blood from which it was prepared, and about 400 times greater than the solids of the red cells, 1 part by weight of the solid per 7,000,000 parts of solution being sufficient to double the rate of CO_2 evolution from the standard bicarbonate-phosphate mixture used by Meldrum and Roughton in testing for catalytic activity. For further details concerning the preparation, reference should be made to the paper by Meldrum and Roughton (1933a) or to the writer's review in Enzymforschung (Roughton, 1934).

A year later Stadie and O'Brien (1933) reported independently the separation from blood hemoglobin of a crude material, which had up to 5 times the catalytic activity of the total solids of the red cells. These authors were at the time of their work unaware of the previous preliminary notes by Meldrum and Roughton. The method adopted by them was of the same type as that of the first stage of one of Meldrum and Roughton's methods: further purification was not attempted.

With technique essentially similar to that of Meldrum and Roughton (1934a), Van Goor (1934) has recently obtained preparations about 3 times more active than the best preparations of Meldrum and Roughton.

Brinkman (1933) has shown that the catalyst can also be isolated from Actiniae by similar procedure.

Biochemical properties of the catalytic material. That the catalytically active substance is enzymic in nature is shown by the following of its properties.

1. It is relatively undialysable: as a rule only small proportions of it pass through the coarser ultrafiltration membranes.

2. The activity is partly or fully destroyed by heating for 30 minutes at 65°C. This is true of laked blood, the crude preparation, and the most highly purified preparation.

3. It is stable over the pH range 4–12, but is destroyed by 30 minutes at pH 3 or pH 13. This is also true of the material, in all grades of purification.

4. The purest material shows most of the usual protein tests.

5. The activity is inhibited partially or completely by typical enzyme poisons, e.g., cyanides (as a rule), sulphides, salts of Cu, Ag, Au, Zn and Hg, and by carbon monoxide.

These observations, some of which have been confirmed by Stadie and

O'Brien (1933), led Meldrum and Roughton (1932) to the conclusion that the catalytically active substance is a typical enzyme: in their paper they give evidence that it is distinct from certain other enzymes known to be present in mammalian red blood corpuscles. That the enzyme is distinct from hemoglobin, and not a product split off from the latter by the procedure of isolation is shown by *a*, the lack of correlation between the distribution of the enzyme and of hemoglobin. This is specially striking in the case of the blood of young goat fetuses, which have been found to have an activity only 1 to 2 per cent of that of the mother blood, although the hemoglobin concentrations of the maternal and fetal blood do not differ by more than a factor of 2.

b. The fact that partial separation of the enzyme from hemoglobin can be effected by comparatively mild processes, such as crystallisation of the hemoglobin, in the case of horse and rat blood (in these species the hemoglobin crystallises very easily), and fractional ultrafiltration (see later).

These facts, though not exhaustive, encouraged Meldrum and Roughton (1932) to the belief that the catalytically active substance is a new enzyme, distinct from any previously described enzyme or system, and they therefore chose a name for it—carbonic anhydrase. This name was actually suggested by Dr. P. Eggleton. Although it focusses attention mainly on the acceleration of CO_2 formation from carbonic acid by removal of water, yet since carbon dioxide is the anhydride of carbonic acid, and since enzymes are often named by the substrate on which they act (e.g., esterases act on esters, lipases on lipoids, etc.), the name chosen does give some slight suggestion that the enzyme also accelerates the formation of H_2CO_3 from CO_2 and H_2O, as is indeed thermodynamically necessary. This reverse action has been abundantly demonstrated by experiment, and is probably of almost equal physiological service to the organism.

The unit of enzymic activity. Meldrum and Roughton (1933a) measured the activity of the enzyme by its effect on the rate of CO_2 evolution from a mixture of 2 cc. of M/5 phosphate buffer, pH 6.8 with 2 cc. of M/5 $NaHCO_3$ (pH 9) when this is violently shaken in a suitable vessel. The CO_2 was evolved into a gas phase of c. 40 cc. and the pressure increase therein measured manometrically at intervals of 15 seconds. Their definition of the enzyme unit was based largely on the simplest conditions of measurement, compatible with accuracy. It is as follows:

Let R_0 = reciprocal of the time taken for the *second* quarter of the CO_2 to be evolved, when no catalyst is present.

R = reciprocal of the time taken in presence of catalyst:

Then unit amount of enzyme is said to be present in the 4 cc. of phosphate-bicarbonate mixture when $(R - R_0)/R_0 = 1$ at 15°C.[2]

Experiments with different amounts of added enzyme showed that $R - R_0)/R_0$ is proportional to the enzyme concentration, unless the latter is so high that diffusion limits the rate of CO_2 evolution in the gas phase. This happened in their apparatus when $(R - R_0)/R_0 > 3$.

R_0 and R are obviously proportional to the velocity constant $k_{H_2CO_3}$ in presence and absence of enzyme respectively. Stadie and O'Brien (1933) also found a proportionality between enzyme concentration and the effect on the velocity constants $k_{H_2CO_3}$ and k_{CO_2}.

Stadie and O'Brien comment on the observation that the effect on k_{CO_2} was, in case of one preparation, 2.6 times greater than the effect on $k_{H_2CO_3}$. This may well have been due to a difference of pH between the two experiments in which k_{CO_2} and $k_{H_2CO_3}$ were respectively measured. The pH activity curve of the enzyme has not yet been published, but there is reason to believe that it has an optimum in the region of pH 7.0. Stadie and O'Brien's experiments on k_{CO_2} were done at pH 7.0 — the pH in the case of the $k_{H_2CO_3}$ experiments is not quoted but since acetate buffer was present in the solutions it was probably less than 6.0.

Distribution of the enzyme in mammalian fluids and tissues. The study of the distribution of the enzyme in the various fluids and tissues is important as regards the general problem of CO_2 transport in the body. Our knowledge on this head, which is mainly due to Brinkman, Margaria, Meldrum and Roughton (1932, 1933) is not yet complete.

1. *Blood.* No trace of the enzyme could be found in the oxalated plasma of human, goat or rabbit blood, provided that allowance is made for the very slight amount of laking of the red cells, which it is almost impossible to avoid, when separating off the plasma. The whole of the enzyme seems to be in the red cells.

The amounts present in the whole blood of various mammals are listed in table 3.

There is an appreciable variation between different individuals of the same species; the most striking feature of the table, however, is the great discrepancy between mother goats and goat fetuses. In the youngest fetuses the amount present in the blood was found to be only 1 per cent of the amount in the mother's blood, and the proportion did not rise much until near the end of pregnancy.

[2] This unit is only a provisional one, and will probably be replaced by a better one when the kinetics of the enzyme reaction have been more fully worked out. One objection is that M/5 $NaHCO_3$, at pH 9.0, is not stable, but slowly loses CO_2 to the air.

Barcroft et al. (1933) have shown that in young fetuses, the volume of blood in the placental vessels is a very large fraction of the total fetal volume, and therefore that the time spent by each red cell in the placenta must be very long. There would therefore be much less need for carbonic anhydrase to aid in the elimination of CO_2 from the young fetus than from lung-breathing animals. As regards the uptake of CO_2 by the blood in the fetal capillaries, the situation is, however, obscure.

Van Goor (1934) reports that the enzyme content of the blood from the umbilical cord immediately after birth is only about half that of normal adult blood.

TABLE 3

Units of enzyme per cubic millimeter of whole blood of various mammals

ANIMAL	NUMBER OF CASES	E/CUBIC MILLIMETER BLOOD	AVERAGE E/CUBIC MILLIMETER BLOOD
Adult goat (anesthetized)	11	1.30–6.10	2.80
Fetus of goat	9	0.01–0.10	Depends on age
Ox (defibrinated)	6	0.06–1.80	1.10
Man	8	0.37–0.68	0.55
Rabbit	2	0.71–1.72	1.21
Goat (normal)	1	1.35	1.35
Horse	1	0.64	0.64
Rat	1	1.70	1.70
Whale	1	1.40	1.40

2. *Other fluids.* No enzyme has been detected in normal milk bile or urine. There is a small amount present both in rabbit, and in human sperm.

3. *Muscle.* Small amounts are present in watery extracts of mammalian muscles, even when these had been previously perfused with saline solution to remove the contained blood.

4. *Pancreas.* Brinkman (1933) reports very appreciable amounts in extracts of the pancreas both in frogs and in man. This is confirmed by Van Goor (1934) who states that the amounts in pancreatic extracts, especially if fresh, may be greater than in the blood. Preliminary experiments (Roughton) have failed to reveal any in the pancreatic juice.

5. Extracts of the following organs gave negative results (Van Goor 1934) lung, heart, kidney, gall bladder, intestinal wall, peripheral nerves. Traces were found in the central nervous system, liver, spleen. In the

last two cases the extracts were not blood-free, and much of their catalytic activity may have been due to contamination with carbonic anhydrase from the blood.

Physiological rôle of carbonic anhydrase in CO_2 output. In appraising the part played by carbonic anhydrase in accelerating CO_2-output in the lung, it will be convenient to assume provisionally the conclusion, which is tentatively reached in the next section on the state of CO_2 in the blood, namely, that:

Of the total CO_2 liberated from the blood in passing through the lungs (subject at rest) about

70 per cent comes from bicarbonate ions
10 per cent comes from pre-formed dissolved CO_2[3]
20 per cent comes from carbamino compounds of CO_2 with hemoglobin

Of the 70 per cent, which comes from bicarbonate, only one-hundredth or less can be furnished per second directly by the plasma (see table 2) since this contains no appreciable carbonic anhydrase. For practical purposes *all* the bicarbonate, which gives rise to CO_2 in the lung must consist of bicarbonate *a*, which was present in the red cells before the blood reached the lung, and *b*, which migrated from the plasma into the red cells whilst the blood was passing through the lung capillaries.

1. The first question to consider is whether there is sufficient carbonic anhydrase in the red cells to enable all this bicarbonate to be hydrated to CO_2 in the time available. Table 3 shows that the average enzyme content of human blood is 0.55 unit per cu. mm. of whole blood, i.e., about 1.3 unit per cu. mm. of red cells. Prima facie this would be sufficient to accelerate the reaction about 5000 times at 15°C., and about 1500 times at 38°C. (the effect of the enzyme becomes relatively less as the temperature is raised) as against a figure of the order of 150 times, which the calculations summarised in table 2 would seem to require. The normal enzyme concentration of the red cells is apparently in comfortable excess of minimum requirements. Two reservations must, however, be made:

a. That the relation between the concentration of the enzyme and its catalytic effect remains linear up to very high concentrations of enzyme.

b. That the activity of the enzyme is the same in its "in vivo" habitat—the red cell—as is its activity in solution.

No knowledge exists at present in regard to *a*, but it is quite possible

[3] The amount from pre-formed H_2CO_3 is only about 0.002 per cent and is therefore negligible.

that at really high concentrations of enzyme the catalytic effect lags behind the concentration: the writer hopes to investigate question *a* by means of the Hartridge-Roughton rapid reaction velocity technique (1923), since with enzyme concentrations of the order found in the red cell, the reactions may at physiological pH only require times of the order of 0.001 to 0.01 second for half completion. Such observations would be of interest also from the general standpoint of enzyme kinetics, since enzyme catalysed reactions of this order of rapidity have never been examined before. Question *b* also has not been investigated—some notes in regard to it will be found later.

2. The bicarbonate, which is already present in the red cells when the blood reaches the lung capillary, can presumably obtain access readily enough to the carbonic anhydrase: but what of the bicarbonate which migrates from the plasma into the red cells during the passage of the blood through the lung capillary? Comparison of the CO_2 dissociation curves of whole blood plasma (true and separated), and of hemoglobin solutions suggests to the writer that not more than a third of the CO_2 formed from bicarbonate in the lung, can have come from bicarbonate which was already present in the red cells when the blood reached the lung. The remaining two-thirds, representing as it does some 50 per cent of the total CO_2 evolution in the lung, must therefore have reached the carbonic anhydrase by migration inwards from the plasma.[4]

According to 1928 views, which in this respect there is no reason to modify, bicarbonate ions can only migrate into the red cell, if chlorine ions come out in exchange. The rate at which the bicarbonate ions of the plasma can be brought into contact with the carbonic anhydrase of the red cells would therefore seem to be conditioned by the speed of the Hamburger "chloride" shift. This process has often been spoken of as "secondary buffering" in that its function is to share out the superior buffering and CO_2 carrying-power of the red cell with the plasma, i.e., to carry out an internal readjustment within the blood. It now leaps into prime importance in the mechanism of CO_2 transport, since about 50 per cent of CO_2 evolved from the blood during its passage through the lung capillary, depends upon it. As to its actual speed, the only data are those of Dirken and Mook (1931), who mixed a suspension of de-

[4] The rate of migration of H_2CO_3 from the plasma into the red cell must almost certainly be too slight to be worth consideration, since the concentration gradients would be so extremely minute. This is confirmed by the experiments on dilute red cell suspensions, mentioned below, in which no appreciable catalytic activity was found.

fibrinated ox red cells with CO_2 saturated serum (hemoglobin-free) in a modified Hartridge-Roughton reaction velocity apparatus, and followed the change in concentration of dissolved CO_2, bicarbonate and chloride ion concentration in the fluid outside the red cells. The figures given by these authors, show that in their experiments at room temperature (c. 15°C.) the bicarbonate-chloride interchange was 90 per cent complete in about 1.3 second. As the authors point out, the condition of the red cells, especially as regards their membrane permeability, may not be the same as in vivo. The matter obviously requires further investigation, but it is difficult at the moment to see how the last point can be controlled. It is indeed possible that there may, in pathological and indeed in normal conditions, be variations in the permeability of the red cell membrane to $H\overline{CO_3}$ and $\overline{Cl}$, with consequent variation in the rate at which CO_2 can be evolved from the blood.

The views, which have just been put forward, have not previously been published—some of them have indeed been developed by the writer whilst preparing this review; they presuppose that the carbonic anhydrase is located inside the red cell, and that no catalysis of the reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$ occurs at the external surface of the red cell. This assumption is supported by experiments of Meldrum and Roughton (1933a) on the catalytic activity of dilute suspensions of red cells. Provided the blood from which the suspensions were prepared was freshly drawn from a vein and then oxalated or defibrinated, the activities found by these authors were less than 0.1 per cent of the activities of corresponding amounts of the same blood when laked. This effectively excludes any appreciable catalysis occurring at the surface of the red cell membrane: furthermore, in dilute suspensions of red cells, owing to the relatively slight volume of the red cells as compared with the outside fluid, only a minute amount of bicarbonate can penetrate into the red cells by exchange with ions from the interior of the latter—hence this mechanism, the importance of which in facilitating the access of plasma bicarbonate to carbonic anhydrase in whole blood has been emphasized above, is rendered practically inoperative in red cell suspensions. These considerations do in fact constitute a serious obstacle to the direct investigation of the enzymic behaviour of carbonic anhydrase in the intact red blood corpuscle.

Physiological rôle of carbonic anhydrase in CO_2 uptake. It is probable that it is dissolved CO_2 that passes mainly through the blood capillary membranes rather than H_2CO_3 molecules or HCO_3 ions. At the existing pHs the H_2CO_3 concentration would be too small for appreciable dif-

fusion of H_2CO_3 to occur: $H\overline{CO_3}$ ions would only be able to diffuse through the capillary membrane into the blood stream, if they brought with them ions of the opposite sign, e.g., $\overset{+}{H}$, $\overset{+}{Na}$ or $\overset{+}{K}$ or exchanged with ions of the same sign, e.g., $\overline{Cl}$ from the blood. Now the concentration of $\overset{+}{H}$ ions is so slight both in the tissue cells and in the blood, that it is very unlikely that they would diffuse out fast enough: on the other hand there is no evidence that the uptake of carbon dioxide by the blood in passing through an organ involves either the uptake of an equivalent amount of metal (sodium or potassium), or the release from the blood of an equivalent amount of some anion such as $\overline{Cl}$.

Let it be assumed, therefore, that dissolved CO_2 molecules in the main enter the blood stream in the tissue capillaries. What happens to them on arriving in the plasma? The figures given in table 2 apply to CO_2 uptake, as well as to CO_2 output, and therefore only a proportion of the order of 1 per cent of the entering CO_2 can be converted directly into bicarbonate in the plasma, since here the reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$ is unaided by the enzyme. The vast majority of the dissolved CO_2 must pass unchanged through the plasma into the interior of the red cell, where in the presence of the abundant supply of carbonic anhydrase, it would hydrate quickly enough[5] to carbonic acid, from which bicarbonate ions would be at once split off. It is clear that unless the increased bicarbonate in the red cell can exchange sufficiently fast with chloride ions from the plasma, the total CO_2 uptake by the blood during the second which it spends in the capillary, will be considerably cut down: in CO_2 uptake the speed of the "chloride shift" (but in the reverse direction) is thus as important as in CO_2 output.

Most vertebrate organs contain only very slight amounts of carbonic anhydrase. This is quite understandable if it is CO_2 that is itself produced in the tissues in the last stage of metabolism. Were there to be abundant carbonic anhydrase present in the tissues, the CO_2 would quickly be hydrated to bicarbonate, and since the latter is not able to diffuse out of the cells so rapidly the rate of CO_2 elimination would be slowed down. In such a location carbonic anhydrase would be an enemy to the organism, rather than a friend: in its absence the CO_2 is able to diffuse away rapidly without loitering appreciably by the wayside in the form of bicarbonate ions. Brinkman's finding that the pancreas contains large amounts of carbonic anhydrase is not out of harmony with this view, since the pancreas excretes not only CO_2 but also large amounts of bicarbonate in the pancreatic juice.

[5] Subject to the reservations mentioned in the previous subsection.

This theory that carbonic anhydrase may obstruct the diffusion away of CO_2, when the latter is produced by some reaction other than $H_2CO_3 \rightleftharpoons CO_2 + H_2O$, has been well exemplified by manometric experiments of Krebs and Roughton (1934) on the rate of liberation of CO_2 from urea in the presence of urease. If a suitable initial pH was chosen, i.e., c. 6.8, there was at first a very large rapid output of CO_2 into the gas phase: on continued shaking most of this CO_2 was reabsorbed and at the final end-point of the process the pressure of CO_2 in the gas phase was

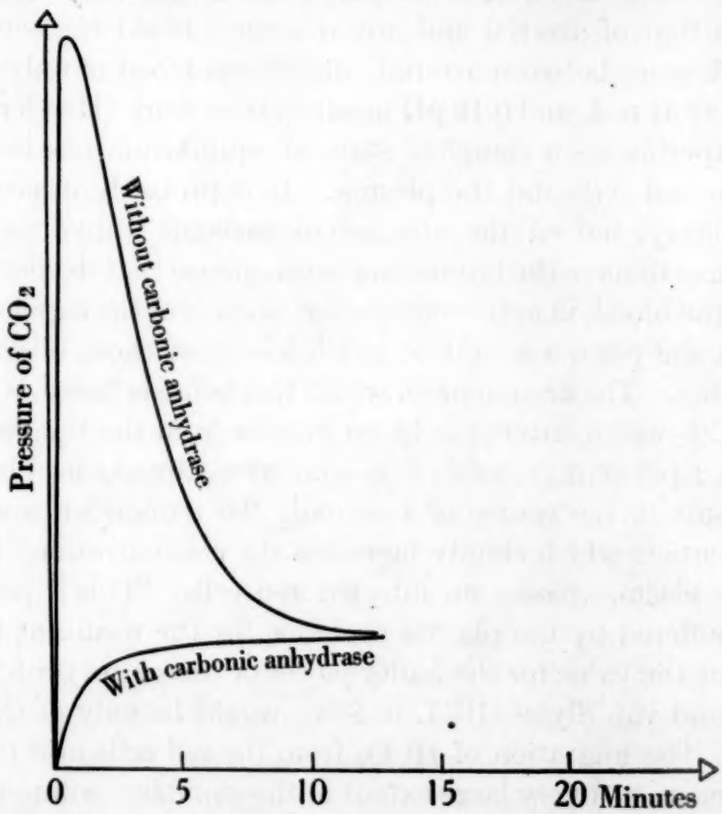

Fig. 2. The effect of carbonic anhydrase upon the time course of evolution of carbon dioxide from urea, under the action of the enzyme urease.

only about 20 per cent of the rapidly developed pressure in the early stage. If carbonic anhydrase was added, the same end-point was reached but there was no initial overshoot: in the latter case the CO_2 liberated by action of urease on the urea changes over into the equilibrium concentration of bicarbonate before it has time to escape into the gas phase, whereas in the former case the initial rate of CO_2 production and rate of escape into the gas phase are much quicker than the velocity of the reaction $CO_2 + H_2O \rightarrow H_2CO_3$ in absence of catalyst (fig. 2).

Similar examples have also been given by Ferguson and Roughton (1934a).

Some consequences and advantages of carbonic anhydrase being situated in the red cell and not in the plasma. i. The "1928" view of CO_2 carriage by the blood maintained that the reaction bicarbonate $\rightleftharpoons$ dissolved carbon dioxide occurred principally in the red cell. It would, therefore, obviously be advantageous to have an abundant supply of the enzyme, which catalyses this reaction, located at the same spot.

ii. Comparison, in vitro, of the pH of blood equivalent in O_2 and CO_2 content to that of arterial and mixed venous blood respectively, indicates a difference between arterial and venous blood of only about 0.03 pH, subjects at rest, and 0.10 pH in subjects at work (Henderson, 1928). In such experiments a complete state of equilibrium has been reached both in the red cells and the plasma. It is probably otherwise in the blood capillary: indeed the absence of carbonic anhydrase from the plasma seems to have the interesting consequence that during the second or so that the blood, in active circulation, spends in the capillary, the pH changes in the plasma would be much less than those observed in "in vitro" studies. The argument on which this belief is based, is as follows:

Of the CO_2 which enters the blood plasma from the tissues, probably only about 1 per cent (v. table 2) is converted directly into bicarbonate in the plasma in the course of 1 second: the remainder, apart from a small percentage which simply increases the concentration of dissolved CO_2 in the plasma, passes on into the red cells. This 1 per cent can easily be buffered by the plasma proteins, for the resultant fall in pH, if we assume the value for the buffer power of the serum proteins quoted by Peters and van Slyke (1931, p. 895), would be only of the order of 0.005 pH. The migration of $H\overline{CO}_3$ from the red cells into the plasma, even if it occurs to a very large extent in the capillary, will not make the pH of the plasma more acid, provided that there is a strict exchange of chloride ions for bicarbonate ions. The final, more acid value of the pH will only be reached in the plasma when there has been time for the equilibrium of the reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$ to be attained in the plasma. This, however, should not have occurred for some seconds after the blood has left the capillary. Similar arguments suggest that in the lung capillaries, the changes in the plasma pH would be equally slight as a result of CO_2 evolution. If this reasoning is correct, the pH of the blood plasma during the actual time when it is in most intimate contact with the delicate tissues of the body, is regulated even more perfectly than had hitherto been thought.

In the red cells, however, it is probable that the variation of pH, whilst the blood is travelling through the capillary, may be greater than has been supposed from in vitro experiments. Owing to the corresponding effect on the oxyhemoglobin dissociation curve this would have the consequence of facilitating both the unloading of oxygen from the blood in the tissues, and the acquisition of oxygen by the blood in the lungs.[6]

iii. Another advantage of having the enzyme in the red cell is that it is probably better protected there from loss by chemical inactivation or by simple leakage away through the walls of the blood vessels. In unpublished experiments Roughton and Winton (1934) have found that, when defibrinated blood, containing both hemoglobin and carbonic anhydrase in the serum, is perfused through the dog's kidney, both hemoglobin and carbonic anhydrase may be lost into the urine, but the enzyme leaks out to a much greater extent than the hemoglobin. Such fractional ultra-filtration "in vivo" may possibly help in the preparation of hemoglobin free from carbonic anhydrase—a study of the properties of hemoglobin free from enzymes is certainly very desirable. Brinkman also (1933, private communication) informs the writer that purified carbonic anhydrase, when injected intravenously into rabbits, disappears rapidly from the blood.

SECTION 2. THE STATE OF CARBON DIOXIDE IN BLOOD. Since 1928, several types of method have been used to investigate whether any of the bound CO_2 of the blood is present otherwise than as free $H\overline{CO}_3$ ions. Consideration shows that these methods fall into two categories, according to whether they attempt to measure the whole, or some specific fraction of the bound CO_2 which is present otherwise than as free HCO_3.

1. Methods which attempt to determine the whole of the bound CO_2 present otherwise than as free $H\overline{CO}_3$. For sake of abbreviation this may be called the x-bound CO_2.

a. Osmotic methods. These depend on the principle that the x-bound CO_2 should make no contribution to the total osmotic pressure of the blood. This has been investigated, by measuring in the presence of CO_2

(i) The lowering of the vapour pressure of blood (Margaria, 1931).

(ii) The lowering of the freezing-point of blood (Stadie and O'Brien, 1931; Stadie and Sunderman, 1931).

[6] This matter has indeed a bearing on the computation of the diffusion constant of the lung to oxygen, and also on the significance of measurements of the oxygen tension of blood drawn directly from arteries. The reviewer hopes to deal with these questions in a later publication.

(iii) The total osmotic pressure of the interior of the red cell (Henriques, 1933).

(iv) Direct determination of the osmotic pressure of hemoglobin solutions containing CO_2 (Adair, 1925).

b. Electrometric methods: These depend on the principle that the x-bound CO_2 would not exert the electrochemical effects to be expected of free $H\overline{CO}_3$ ions. This has been investigated by measurements of

(i) The pK_1' of the Henderson-Hasselbalch equation in hemoglobin solutions (Stadie and Hawes, 1928; Margaria and Green, 1931).

(ii) Membrane equilibria between hemoglobin solutions containing CO_2 and their dialysates [Henriques (1928), Adair (1928, 1934a, b)].

(iii) The titration curve of sodium hemoglobinate in the pH range 6.0 to 8.0, the titrating acid being in one case HCl and in the other CO_2

2. Methods which attempt to determine a specific fraction of the x-bound CO_2. This may not represent the total x-bound CO_2.

a. Kinetic methods—depending on the rate of uptake or output of CO_2 from hemoglobin solutions or blood, in which the enzyme, carbonic anhydrase, is deficient or inactive (Henriques, 1928; Meldrum and Roughton, 1933b).

b. Analysis of the residual CO_2 in hemoglobin solutions, which is not precipitated by barium at alkaline pH (Meldrum and Roughton, 1933b; Ferguson and Roughton, 1934a, b).

Reasons are given below for believing that methods 2a and 2b both estimate the CO_2 which is bound to hemoglobin in the form of carbamino compounds. Preliminary evidence suggests that the total x-bound CO_2 may be greater than that which can be accounted for by carbamino-compounds. For convenience we shall refer to the hypothetical excess of x-bound CO_2 over carbamino-bound CO_2 as y-bound CO_2. The various states of CO_2 in blood, if these views are correct, are then related to one another in the manner summarised in figure 3.

Since the evidence in regard to the carbamino-bound CO_2 seems to be more complete, from the physiological point of view, and also to be freer from doubt on theoretical counts, we shall first review the recent work on this subject, and then pass on to the description of the work on x-bound CO_2, and the possible existence of y-bound CO_2.

CARBAMINO-COMPOUNDS OF CO_2 WITH HEMOGLOBIN. This subject may be introduced by considering in more detail Henriques' (1928) results on the rate of evolution of CO_2 from hemoglobin solutions when

shaken violently in vitro. The curves obtained by him are divisible rather sharply into

(i) A rapid phase of 5 seconds' or so duration in which a large portion of CO_2 came off.

(ii) A prolonged slow phase, in which the remainder of the CO_2 came off at about the same rate as from serum or from phosphate-bicarbonate mixtures of physiological pH.

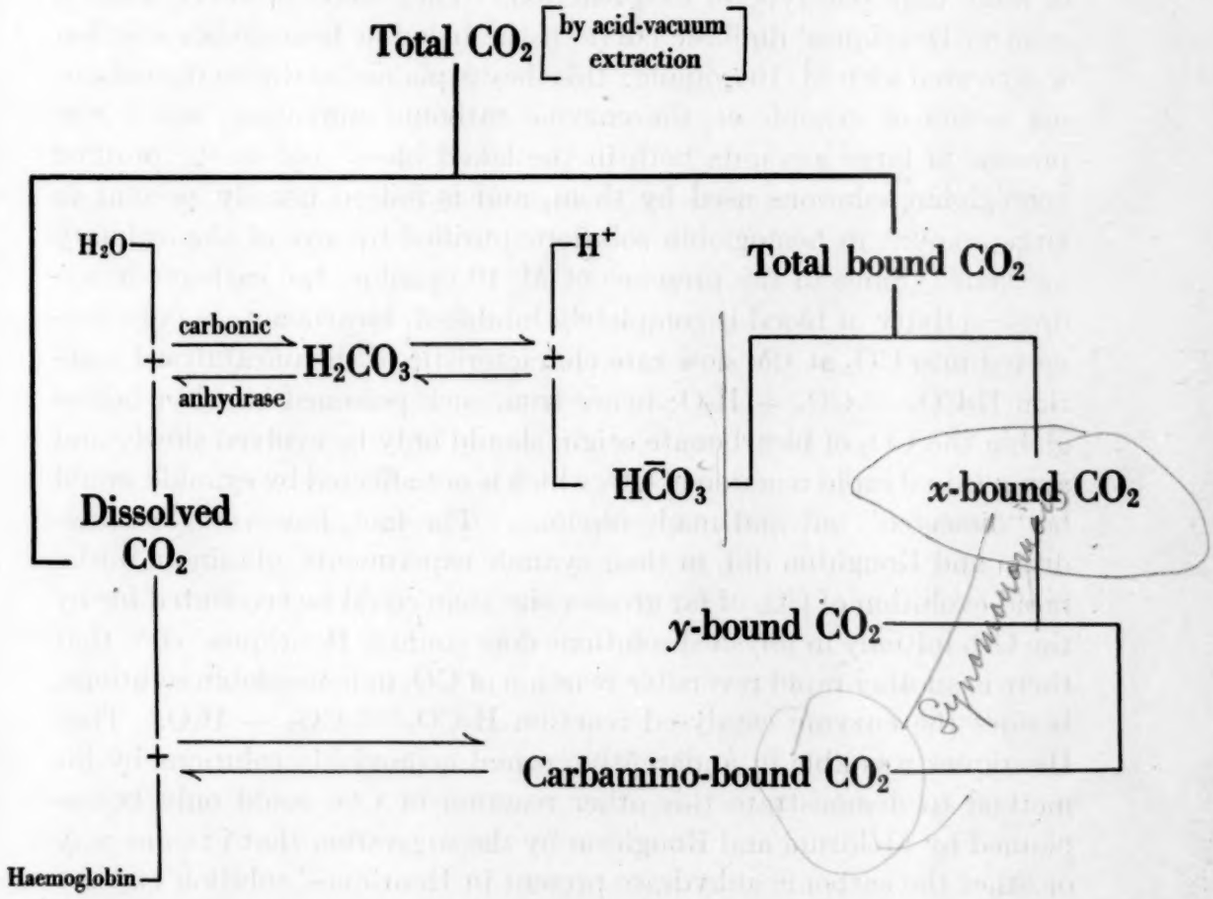

Fig. 3. Various forms in which carbon dioxide is believed to be present or combined in blood, and their inter-relationships.

Henriques thought, quite naturally, that the sudden rapid evolution of CO_2 could not be explained by the presence in the hemoglobin solutions of a catalyst for the $H_2CO_3 \rightleftharpoons CO_2 + H_2O$, reaction since there seemed no conceivable reason why such a catalyst should only act for five seconds and then stop acting. He attributed it, on the other hand, to the rapid dissociation of a reversible compound between CO_2 and hemoglobin, for which he revived Bohr's name "carbhemoglobin."

Meldrum and Roughton (1933b), on repeating Henriques' experiment, were unable to obtain the diphasic curve either with laked blood or with hemoglobin solution, prepared, as far as possible, in the same way as in Henriques' work (by washing horse red cells in sugar solution, laking with the saponin, and then removing the "detritus" by centrifuging). They found, on the other hand, that the whole of the CO_2 was evolved rapidly in a smooth manner, and that there was thus no evidence of more than one type of CO_2 reaction. They were, however, able to confirm Henriques' diphasic curve, if the blood or hemoglobin solution was treated with M/10 cyanide: this they explained as due to the poisoning action of cyanide on the enzyme carbonic anhydrase, which was present in large amounts both in the laked blood and in the purified hemoglobin solutions used by them, and is indeed usually present in large amount in hemoglobin solutions purified by any of the ordinary methods. Since in the presence of M/10 cyanide, the carbonic anhydrase activity of blood is completely inhibited, bicarbonate is only converted into CO_2 at the slow rate characteristic of the uncatalysed reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$: hence from such poisoned blood or hemoglobin the CO_2 of bicarbonate origin should only be evolved slowly and any residual rapid reaction of CO_2 which is not affected by cyanide would be "dissected" out and made obvious. The fact, however, that Meldrum and Roughton did, in their cyanide experiments, obtain an initial rapid evolution of CO_2 of far greater size than could be accounted for by the CO_2 initially in physical solutions does confirm Henriques' view that there is another rapid reversible reaction of CO_2 in hemoglobin solutions, besides the enzyme catalysed reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$. That Henriques was able in apparently normal hemoglobin solutions by his method to demonstrate this other reaction of CO_2 could only be explained by Meldrum and Roughton by the suggestion that in some way or other the carbonic anhydrase present in Henriques' solution had got poisoned or else eliminated.

Henriques showed that the new reaction occurred more readily in reduced hemoglobin solutions than in oxyhemoglobin solutions; it is difficult, however, to deduce from his results just what rôle the reaction plays in physiological processes, since the experiments were done at 18°C. and the pH was not stated.

In regard to its nature, Henriques first suggested that the reaction was of a carbamino kind, i.e., a reaction between CO_2 and the $-NH_2$ groups of the protein thus

$$CO_2 + PNH_2 \rightleftharpoons PNHCOOH \leftrightharpoons PNH\overline{C}OO + \overset{+}{H} \qquad (7)$$

The physical chemistry of this type of reaction is now fairly well-known, thanks to the researches of Siegfried (1905), Faurholt (1925), and Meldrum and Roughton (1933b). Faurholt in particular has made a very full and convincing study of the mechanism of the carbamino equilibrium for the case of several simple amines, namely ammonia, the methylamines and glycine. A detailed study of his writings is indispensable to any investigator who wishes to undertake further work on the carbamino compounds, whether of simple amines or of complex $-NH_2$ containing substances such as proteins. Such knowledge has enabled Roughton and his co-workers to bring forward strong evidence for the existence of significant amounts of carbamino bound CO_2 in hemoglobin solutions under physiological conditions. It will be useful to begin by cataloging the relevant properties of the simpler carbamino compounds.

(i) CO_2 combines in the carbamino form with substances containing an $-NH_2$ group, but not with substances containing an $-\overset{+}{N}H_3$ group, e.g., CO_2 combines with ammonia NH_3, methylamine CH_3NH_2 and with the glycinate ion $CH_2NH_2\overline{COO}$; but does not combine with the ammonium ion $\overset{+}{N}H_4$ (as in ammonium chloride solution), the methylamine ion $CH_3\overset{+}{N}H_3$, or neutral iso-electric glycine (the formula for which, according to the modern Zwitterion theory is $CH_2\overset{+}{N}H_3\overline{COO}$).

(ii) No carbamino reaction occurs between amino containing substances and H_2CO_3 $H\overline{CO_3}$ or $\overline{\overline{CO_3}}$.

(iii) The rate of the reaction is intrinsically rapid: there is no evidence at present that there is any enzyme in the body to accelerate it. The amount of carbamino compound formed at any given pressure of CO_2 increases up to a point with pH: this is due partly to the fact that the proportion of the amine substance in the $-NH_2$ form increases with alkalinity at the expense of that present in the $-\overset{+}{N}H_3$ form, and partly to the fact that the reactions shown in equation (7) all proceed further towards the right owing to the depression of the hydrogen ion concentration at alkaline pH.

(iv) The "affinity" of $-NH_2$ groups for CO_2 decreases with rise of temperature.

(v) Carbamino compounds decompose according to equation (7), the reactions shown there proceeding in turn from right to left. The rate of decomposition is extremely slow in alkaline solution, pH > 12, presumably because the reaction $PNHCOOH \rightleftharpoons PNH\overline{COO} + \overset{+}{H}$ is so much displaced to the right: at 0°C. indeed, solutions of the simpler

carbamino compounds are stable for many hours in this range of alkalinity.

As the pH is made more acid the rate of decomposition increases, the overall rate according to Faurholt depending upon the speed of removal of CO_2 from the sphere of action, i.e., (to the form of $H\overline{CO}_3$ and/or $C\overline{\overline{O}}_3$ if the pH > c. 8, and in part or in whole to the form of gaseous CO_2 if the pH is more acid).

(vi) The barium and calcium salts of carbamino compounds are soluble: this gives a simple way of separating carbamino compounds from carbonates in alkaline solution. After addition of excess of $CaCl_2$ or $BaCl_2$, the $CaCO_3$ or $BaCO_3$ precipitate is removed by centrifugation. The salt of the carbamino compound can then be crystallised from the supernatant fluid (Siegfried, 1905), and if desired the amount of carbamino compound in the latter estimated by titration with acid (Faurholt, 1925) or by measuring gasometrically the amount of CO_2 liberated on addition of excess of acid (Ferguson and Roughton, 1934b).

If the blood proteins are to be capable of forming carbamino compounds with CO_2 under physiological conditions, it must first be shown (v. property (i) above) that a significant amount of their amino-nitrogen is in the $-NH_2$ form at pH 7.1–7.4, temperature 38°C. That such is the case follows from the fact that the blood proteins, especially hemoglobin, are good buffers in the physiological range, i.e., mop up $\overset{+}{H}$ ions readily: furthermore the heat liberated per gram ion of $\overset{+}{H}$ so bound is found to be of the order of 10,000 cals. (Roughton, 1930). This indicates that the reaction of hydrogen ion binding is of the type $NH_2 + \overset{+}{H} \rightarrow \overset{+}{N}H_3$, for this is the only type of $\overset{+}{H}$ binding which is known to yield so high a heat of reaction. It can therefore be taken that under physiological conditions the blood proteins, especially hemoglobin, have $-NH_2$ groups *available* to combine with CO_2, and it is thus a priori reasonable that carbamino combination should occur between them.

In 1933 Meldrum and Roughton carried the matter a stage further: instead of following the rate of evolution of CO_2 from hemoglobin, as in Henriques' work, they observed the rate of uptake of CO_2 by blood, which was initially free of CO_2. This, they point out, has great advantages over the procedure of Henriques as regards ease of interpretation and power of varying the conditions over a wide range. Their results and conclusions may be briefly summarised as follows:

When normal, CO_2-free, laked blood is shaken violently with a gas mixture containing CO_2, the whole quota of CO_2 is taken up rapidly.

and it is not possible to separate the uptake process into two or more phases. If the blood is, however, treated with 0.1 M HCN or higher concentrations the CO_2 uptake occurs in two phases, of which the first is rapid and the second is a long slow one, of the same rate as the uptake of CO_2 by buffer solutions (pH 7–8) containing no carbonic anhydrase.

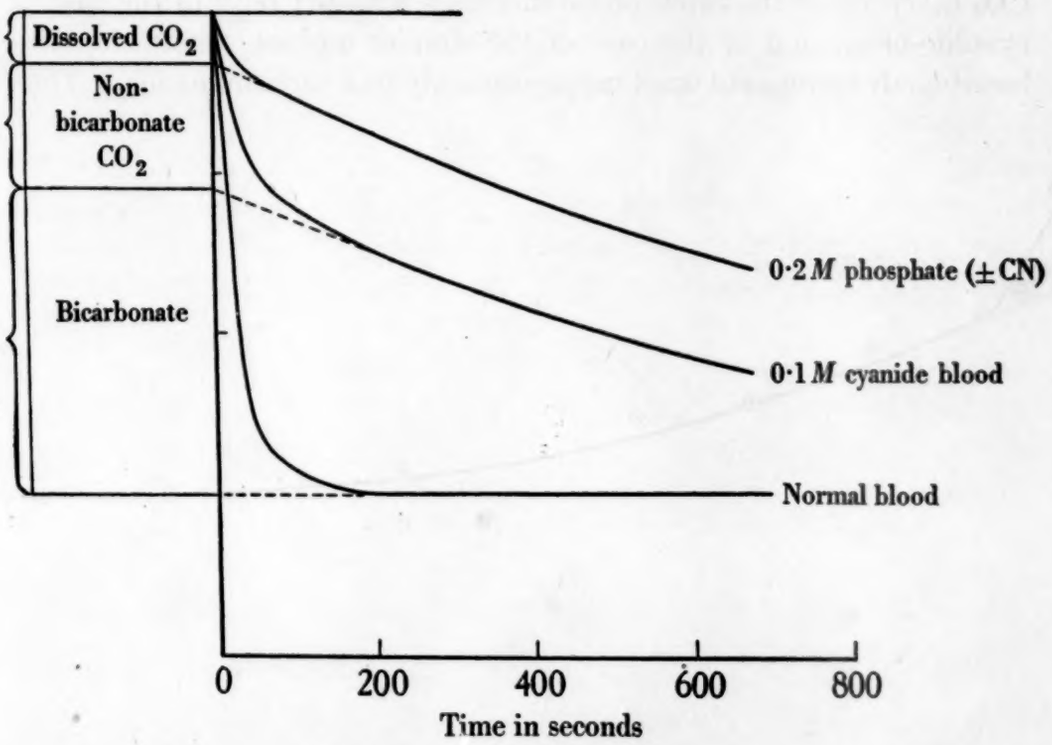

Fig. 4a. Rate of uptake of CO_2 by M/5 phosphate buffer pH 7.4, by blood poisoned with M/10 cyanide, and by normal blood. The ordinates represent the pressure of CO_2 in the gas phase in each case.

For phosphate buffer the rapid initial uptake is due simply to dissolved CO_2, the slow delayed uptake is due to the formation of bicarbonate from CO_2.

For cyanide blood, the rapid initial uptake is due to dissolved CO_2 together with some rapid, non-bicarbonate, chemical compound of CO_2 the slow delayed uptake is due to bicarbonate formation.

For normal blood, the dissolved CO_2, the non-bicarbonate bound CO_2 and the bicarbonate bound CO_2 are all taken up in the rapid initial phase.

The diphasic curve is of the type shown in figure 4A. The slow phase is thus due to the uncatalysed $CO_2 + H_2O \rightarrow H_2CO_3$ reaction: the CO_2 taken up in the rapid phase is considerably more than can be accounted for by physical solution, so that the excess must be due to the formation of some rapid CO_2 compound, which is not carbonic acid or bicarbonate, and which for the moment may be called non-bicarbonate CO_2. The

amount of CO_2 taken up rapidly in this non-bicarbonate form varies from 5 to 20 per cent of the total CO_2 taken up by blood, according to the conditions, and is thus a significant fraction of the whole uptake. Solutions of ammonia, glycine, amino-acids and peptides behave in just the same way as the "cyanide"-blood: the amount of non-bicarbonate CO_2 taken up in the rapid phase increases with pH both in the case of cyanide-blood and in the case of the simpler amines, where the non-bicarbonate compound must unquestionably be a carbamino one. Thus

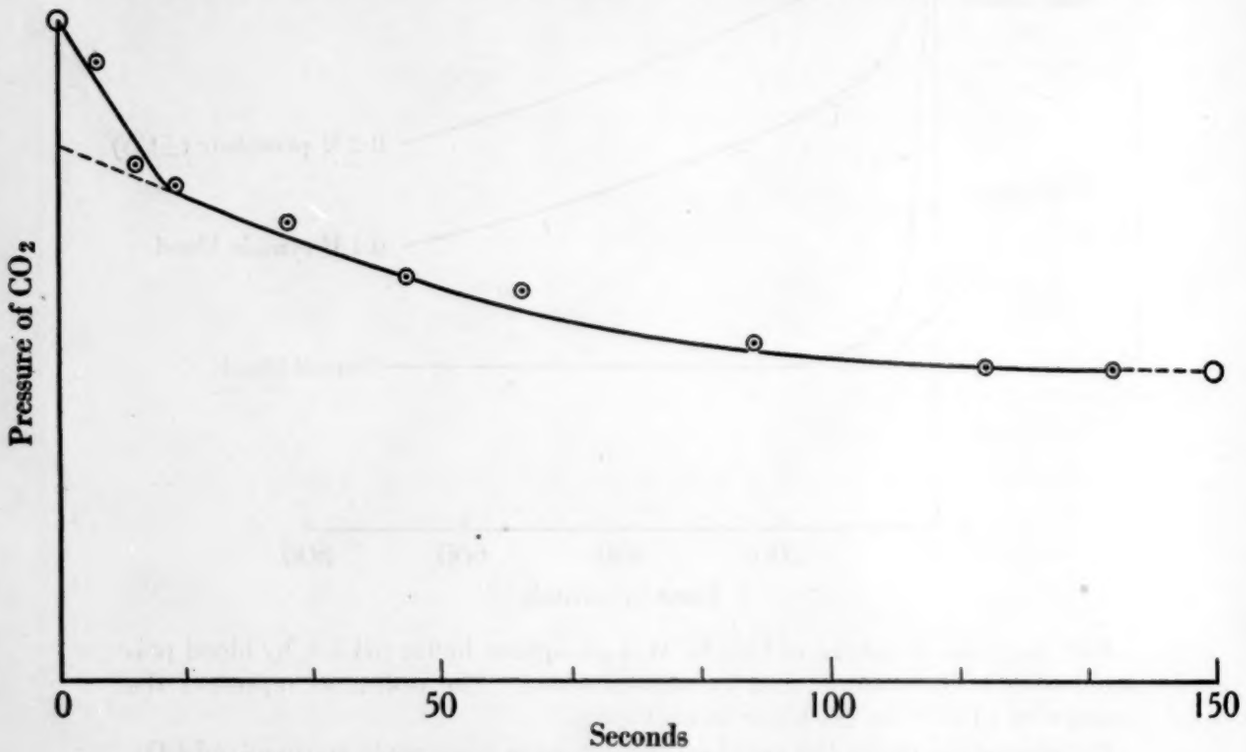

Fig. 4b. The difference between the respective rates of uptake of CO_2 by oxygenated and reduced blood at 37°C., in the presence of M/10 HCN, plotted against time.

the non-bicarbonate CO_2 behaves like carbamino-bound CO_2 in respect to property (ii): rise of temperature also reduces the amount of rapid non-bicarbonate bound CO_2, in the case of cyanide blood, thus bringing blood into line with the simpler amines as regards property (iii) also. That the non-bicarbonate CO_2 is in the main bound to hemoglobin is shown by the effect of oxygenation and reduction: reduced blood takes up much more non-bicarbonate CO_2 than oxygenated blood under otherwise parallel conditions, and so the compound to which the non-bicarbonate CO_2 is bound must be, in the main, one which is affected by

oxygenation. There is no compound, except hemoglobin, in blood which is known to be reversibly affected by oxygenation and it must therefore be hemoglobin to which CO_2 is, in the main, bound in the non-bicarbonate form.

Controls showed that there is every reason to suppose that the same non-bicarbonate binding of CO_2 occurs in normal blood, containing no cyanide, and that the latter has no relevant action other than that of poisoning the abundant carbonic anhydrase normally present in blood. It would be desirable, if possible, to do the same experiments on purified hemoglobin, which, whether by crystallisation or other means, had been rendered free of carbonic anhydrase.

Unpublished experiments of the same kind on plasma (by Roughton) show that the CO_2 taken up in the rapid phase is not greater than can be accounted for by physical solution, and therefore that in plasma, the amount of rapid non-bicarbonate bound CO_2 is, under physiological conditions, negligible. In these experiments there was no need to add cyanide for the plasma (as usual) contained no carbonic anhydrase.

Meldrum and Roughton's experiments needed supplementing in three respects:

a. They gave no indication of whether the non-bicarbonate bound CO_2 resembled carbamino-bound CO_2 as regards properties (v) and (vi) of the list of carbamino-compound properties.

b. The pH values used by them were close to the physiological range but the temperature did not exceed 15°C. No experiments were done at 37°C., because at that temperature the reaction $CO_2 + H_2O \rightarrow H_2CO_3$ in absence of catalyst proceeds too fast for the rate of CO_2 uptake to be clearly resolvable into two distinct phases when studied by manometric technique. Since then, however, Roughton (1934 unpublished experiments) has found that if reduced cyanide blood and oxygenated cyanide blood are respectively shaken under exactly the same conditions with the same gas mixture and the difference in amount of CO_2 taken up is plotted against time a diphasic curve is again obtained even though the CO_2 uptake of neither blood separately is diphasic in character. In the example plotted in figure 4B the rapid part of the difference in CO_2 uptake amounts to about 35 per cent of the total difference in CO_2 uptake between reduced and oxygenated blood; hence Roughton concludes that about 35 per cent of the extra CO_2 carrying power of reduced blood over that of oxygenated blood is due to carbamino-bound CO_2, if the pressure of CO_2 is 30 mm. Hg and the pH is about 7.25 as in the experiment quoted. The conditions are thus close to physiological as regards all the important factors.

c. It would be preferable to confirm the results by some method which did not involve the presence in the blood of the massive amounts of cyanide required in Meldrum and Roughton's technique.

To fill up these gaps, Ferguson and Roughton (1934a) have investigated the possibility of separating and estimating the supposed carbamino compound of CO_2 with hemoglobin by the same analytical technique as has been used by Faurholt in the case of the carbamino compounds of the simpler amines. This, it will be recalled, consists in making the solution suddenly alkaline and then treating at once with excess of $BaCl_2$: the precipitate of $BaCO_3$ is centrifuged down, and the carbamino compound is left in the supernatant fluid, wherein it is very conveniently and accurately measured by Van Slyke's gasometric technique. In applying this method to hemoglobin solutions several special difficulties were met with, of which the most serious were:

(i) Possibility of hematin formation.

(ii) Failure of the $BaCO_3$ precipitate to sediment completely owing to the "protective action" of the protein.

(iii) Possible conversion of some of the dissolved CO_2 originally present in the hemoglobin solution, into new carbamino compounds, when the mixture is suddenly made alkaline.

(iv) Dissociation of some of the carbamino-compound during the estimation process.

Ferguson and Roughton were unable to overcome these difficulties completely, but as a result of special technique and controls they claim to have allowed satisfactorily for errors due to them by means of relatively small blank corrections. The upshot of their work was to show that in hemoglobin solutions equilibrated with CO_2, there is an appreciable amount of bound CO_2 which cannot be precipitated by barium at alkaline pH and which is therefore present in the form of a compound resembling the simpler carbamino compounds as regards the very important property number (vi) on the list. At alkaline pH the compound only dissociates very slowly, but on acidification to pH 6.0 — 7.0 free CO_2 is split off from the compound directly and rapidly: thus in regard to the effect of pH on its stability, i.e., in property (v), this bound CO_2 in hemoglobin solution again resembles the simpler carbamino compounds. Finally the amount of the unprecipitable CO_2 compound was found to vary with pH and temperature in just the same way as did the "non-bicarbonate bound CO_2" of Meldrum and Roughton: it likewise resembles the latter in being much decreased, if the hemoglobin solution is oxygenated instead of reduced. Indeed in conditions close to those of

fig. 4B 30 to 40 per cent of the extra CO_2 taken up by reduced hemoglobin (as compared with oxyhemoglobin) was found to be unprecipitated by barium; this may be compared with the proportion of 35 per cent given by Meldrum and Roughton's method (v. supra). Though comparisons have yet to be made on the same solution by Meldrum and Roughton's technique on the one hand, and by Ferguson and Roughton's technique on the other hand, there can be little doubt that the same compound is being dealt with in the two cases, and since it thus possesses all the properties of simpler carbamino-compounds listed above, Roughton and

TABLE 4

Carbamino-bound CO_2 in hemoglobin solutions under various conditions

OBJECT OF EXPERIMENT	Hb CONCENTRATION IN MM/LITRE	TEMPERATURE	APPROXIMATE pH	pCO_2	TOTAL CO_2	CARBAMINO CO_2
				mm. Hg	*volumes per cent*	*volumes per cent*
Effect of pH	10 (reduced)	38	7.8	14.5	50	3.2
	10 (reduced)	38	8.6	14.5	330	18.2
Effect of temperature	10 (reduced)	20	7.35	44.5	73.2	9.1
	10 (reduced)	38	7.33	46.0	55.3	5.1
Effect of hemoglobin concentration	4.4	38	8.0−	40		5.0
	8.8	38	8.0+	36.2		10.0
Effect of oxygenation	10 (reduced)	38	7.16	35.0	31.8	2 6
	10 (oxygenated)	38	7.10	35.0	21.5	0.7

his co-workers claim that it actually is a carbamino compound of CO_2 with hemoglobin.

Table 4 shows the effect of pH, temperature, hemoglobin concentration and oxygenation upon the amount of CO_2 bound to hemoglobin in the carbamino form, when purified hemoglobin solutions are equilibrated with gas mixtures containing the CO_2, and the "carbamino-CO_2" is estimated by Ferguson and Roughton's method.

Physiological rôle of carbamino compounds. The only data at present as to the rôle of carbamino-bound CO_2 in carbon dioxide transport, under physiological conditions, are those by Ferguson and Roughton (1934b). These authors measured:

a. The total CO_2 taken up by oxyhemoglobin and reduced hemoglo-

bin solutions at 38°C., CO_2 pressure between 30 and 60 mm. Hg, and pH values ranging from 7.0 to 7.4, i.e., the CO_2 dissociation curves of oxygenated and reduced hemoglobin solutions.

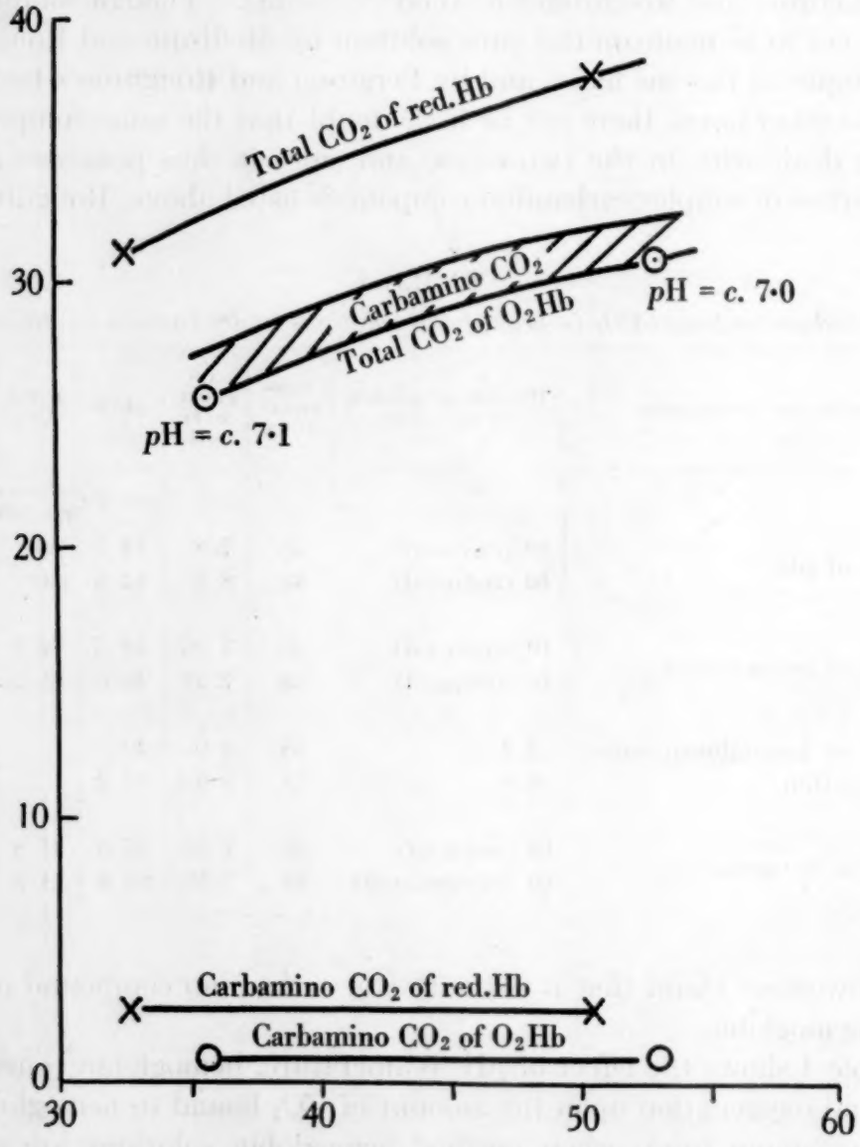

Fig. 5. Amounts of total CO_2, and carbamino bound CO_2 in reduced hemoglobin and oxyhemoglobin solution under physiological conditions of CO_2 pressure, pH and temperature (data of Ferguson and Roughton).

b. The carbamino bound CO_2 taken up by the same solutions under the same conditions. The results of their best experiment are plotted in figure 5. Although the carbamino-bound CO_2 is only a small fraction of the whole CO_2, i.e., 2 to 10 per cent, it is of greater significance

than this in CO_2 transport. For on the basis of figure 5 Ferguson and Roughton calculate that, of the *difference* in CO_2 content between average arterial whole blood and venous blood in man, 15 to 20 per cent at least is due to the difference in carbamino-bound CO_2: whilst, as regards the red cells only, some 40 per cent of their change in CO_2 content during the respiratory cycle is deduced to be due to changes in the carbamino-bound CO_2.

In these calculations it is assumed that the results on purified hemoglobin solutions of one species (ox) are applicable to whole blood of another species (man)—data quoted by L. J. Henderson (1928): it would be desirable to do all the experiments on material of the same species. Another point, which is still left open, is the possibility of appreciable carbamino CO_2 being bound to other proteins in the blood, besides hemoglobin: unfortunately both the plasma proteins and stroma proteins interfere much more seriously with the sedimentation of the $BaCO_3$ precipitate in Ferguson and Roughton's method than does hemoglobin, and hence so far these authors have not been able to bring forward data on whole blood or plasma. In regard to the latter, however, continuation of Meldrum and Roughton's work (see p. 268) suggests that the amount of CO_2 bound to them in the carbamino form is under physiological conditions negligible.

No precise measurements have yet been made of the speed of the carbamino reaction between CO_2 and hemoglobin, but the reaction, like that between CO_2 and simpler amines, is known to be rapid, and is, indeed, probably rapid enough to reach nearly to completion in the capillary. It does not at present seem rash to conclude that, in mammals, some 15 per cent or more of the CO_2 transported by the blood during the respiratory cycle is carried by an alternative mechanism of a carbamino-type in which the enzyme carbonic anhydrase is not involved.

In cases where the latter is deficient, e.g., in fetal blood, the carbamino mechanism might perhaps play an even more important rôle in CO_2 transport: so might it also in the case of cold-blooded animals, since carbamino compounds are more readily formed at low temperatures. These are fields which call for further investigation.

The inter-relationships between oxygen binding and carbamino CO_2 binding by hemoglobin. The hemoglobin molecule, like all protein molecules, contains many hydrogen ions, which dissociate off at various pH levels.

Henderson (1920) and Van Slyke (1922) have suggested, however, that there is one particular hydrogen ion which dissociates from the

neighbourhood of each hematin nucleus in the hemoglobin molecule, and does so more readily if oxygen is attached to the latter. On this account the special hydrogen ion in question has been called the "oxylabile" hydrogen ion. Van Slyke and his co-workers have calculated the value of its ionisation constant for oxyhemoglobin and reduced hemoglobin respectively.

It has usually been thought that the extra CO_2 taken up by reduced hemoglobin solution, when equilibrated with a given pressure of CO_2, over that taken up by the same solution if oxygenated and equilibrated with the same pressure of CO_2, was entirely due to difference in acidic strength of the oxylabile ionising groups. According to Ferguson and Roughton's results, however, 25 per cent or more of the difference is due to more CO_2 being bound to reduced hemoglobin in the carbamino form than to oxyhemoglobin: attempts to calculate the oxylabile ionisation constants of oxyhemoglobin and reduced hemoglobin from determinations, in which CO_2 is present, therefore seem questionable.

Ferguson and Roughton further point out that the oxylabile group at which the special $\overset{+}{H}$ ion combines and dissociates is almost certainly an $-NH_2$ group: they suggest, indeed, that at physiological pH much of the CO_2 bound in the carbamino form, is combined at this particular $-NH_2$ group and that it is for this reason that oxygenation of the hemoglobin molecule so greatly affects the amount of carbamino-bound CO_2. If this argument is true, attachment of CO_2 to the oxylabile NH_2 group should conversely decrease the affinity of hemoglobin for oxygen. CO_2 should therefore have a specific effect on the position of the oxyhemoglobin dissociation curve, apart from that due to the alteration in pH of the solution. Attempts by Barcroft and Murray in 1926 to demonstrate such an effect for the analogous case of the carboxyhemoglobin dissociation curve proved negative: recently, however, in the more accurate experiments of Margaria and Green (1933) it was found that at pH 7.4, the dissociation curve of oxyhemoglobin in the presence of CO_2 is displaced considerably to the right of its position in a solution of NaCl, of the same ionic strength but containing no CO_2.

Ferguson and Roughton's chemical picture may be summarised as follows: it is believed that there is an oxylabile $-NH_2$ group in the neighbourhood of each hematin nucleus of the hemoglobin molecule — that CO_2 and H^+ ions compete with one another for combination with this group—that the affinity for the group both of CO_2 and of $\overset{+}{H}$ is decreased by oxygenation of the hemoglobin molecule and that, finally,

the attachment of either CO_2 or $\overset{+}{H}$ ions to the oxy-labile NH_2 group decreases the affinity of the hemoglobin for oxygen.

THE X-BOUND CO_2 OF THE BLOOD. Consideration must now be given to the x-bound CO_2, which, it will be remembered, was defined as the difference between the total bound CO_2 of the blood and the CO_2 bound in the form of free bicarbonate ions. Of these two quantities the former is readily measurable by exact chemical methods (i.e., by acid and vacuum extraction); in regard to the latter there is unfortunately an obstacle which has not yet been overcome by any of the osmotic or electrometric methods listed at the beginning of this section. All such methods determine, not the true concentration of bicarbonate ions in the free state, but the apparent concentration or "activity" of the ions: to obtain the true concentration $[H\overline{CO_3}]$ therefrom it is necessary to divide by the activity coefficient, i.e.,

$$a_{HCO_3}/f_{HCO_3} = [H\overline{CO_3}] \tag{8}$$

where a_{HCO_3} = activity of bicarbonate ion.
f_{HCO_3} = activity coefficient of bicarbonate ion.

In very dilute solutions of total ionic concentrations c. 0.001 M, the value of f_{HCO_3} is approximately unity, but as the total ionic concentration is raised the value of f_{HCO_3} falls to a minimum, which is probably not yet reached under the ionic strength conditions of physiological fluids. Up to a certain point, the value of the activity coefficient can be theoretically calculated by the equation of Debye and Hückel, which is worked out mathematically from the consideration that in solution an ion of one sign of charge must have, on the average, more ions of the opposite sign of charge in its neighbourhood, than ions of the same sign. The simplest form of Debye and Hückel's equation is as follows.

$$-\log f = 0.5\, \nu^2 \sqrt{\mu} \tag{9}$$

where f = activity coefficient of the ion
ν = valency of the ion.
μ = ionic strength = $\frac{1}{2}\,\Sigma c\nu^2$
$\Sigma c\nu^2$ = sum obtained by adding together concentration × square of valency of each and every ion in the solution.

In solutions containing only crystalloid ions this equation usually holds good up to values for $\mu = 0.01$; for higher values of μ correction terms have been theoretically worked out.

In tests on solutions of strong electrolytes the apparent concentration,

or activity, of given ions has been determined by osmotic or electrometric methods and the activity coefficient calculated therefrom by dividing by the total concentration of the ion as measured chemically. The agreement of the activity coefficient so obtained with the values as calculated from the equations, not only confirms the validity of the Debye-Hückel theory, but also indicates that in these cases the whole of the ions concerned are in the free state.

Unfortunately, the Debye-Hückel equations cannot at present be rigorously applied if proteins are present in the solution under consideration, for there is no theoretical information as to the effect which a large protein molecule (containing, as it does, a mosaic of numerous electric charges) would have on the inter-ionic forces, and hence on the activity coefficients. It is true that some have held that the charges on the protein are so far apart that they exert practically no activity effect, or at most an effect only equal to that which would be exerted by univalent ions present in such concentration that the total charge per unit volume due to them would be equal to the total number of charges on the protein contained in unit volume. Others, however, believe that from the activity point of view, proteins may behave like multivalent electrolytes, and thus exert a large effect on the ionic strength of the solution, and hence on the activity coefficients. Until the activity "effect" due to the protein is known under the precise conditions under study, no one can say certainly whether an observed lowering of the apparent concentrations of given ions, e.g., Na and Cl, in the presence of protein is due entirely to the activity effect of the protein, or whether it is in part, or perhaps in whole, due to direct combination of the ions in question with the protein.

The uncertainty of the activity coefficient corrections in protein solutions is thus a bar to any rigorous calculation of the x-bound CO_2 or even to an approximate calculation thereof, for in the researches reviewed below an error of 10 per cent in the activity coefficient assumed for the $H\overline{CO}_3$ ion might easily have been made, and such an error would have a very serious effect on the computation of the x-bound CO_2. Furthermore in most of these researches there are also individual points of special difficulty which will be mentioned as each research is dealt with in turn. In spite of these pessimistic warnings it is, however, necessary to review the evidence in some detail for (i) no review on recent work on CO_2 transport would be complete without such a treatment (ii) in certain instances the effects observed are beyond what might be reasonably attributed to activity effects of the proteins. In these

cases the most plausible, and probably the most profitable, hypothesis is that the x-bound CO_2 consists not only of CO_2 bound to hemoglobin in the carbamino form, but also of $H\overline{CO}_3$ and/or CO_2 bound to hemoglobin in some other form, which has for convenience been called above the y-bound CO_2.

a. Osmotic pressure methods. (i). The lowering of the vapour pressure. Margaria (1931) equilibrated defibrinated blood at 18°C. with air containing (α) 0.4 per cent CO_2 (β) 10 per cent CO_2, and compared the extra amount of total CO_2 taken up (as estimated by Van Slyke methods) with the decrease in vapour pressure (by Hill's (1930) thermoelectric method). He found that the change in vapour pressure was only from 55 to 65 per cent of what would be expected if the whole of the extra CO_2 taken up was osmotically active, and that both red cells and purified hemoglobin solution behaved in this way also, the observed discrepancy in the latter case increasing with hemoglobin concentration. On the other hand, for blood serum the changes in combined CO_2 and vapour pressure were within the limits of experimental error equivalent.

In experiments with hemoglobin containing NaCl or $NaHCO_3$, not equilibrated with CO_2, the decrease in vapour pressure was 95 per cent or more of that expected from the concentration of salt added to the hemoglobin. From these observations, Margaria concluded that the observed discrepancy was not due to a lowering effect of hemoglobin on the activity coefficient of the $H\overline{CO}_3$ ion, but rather to the formation of appreciable amounts of a direct, osmotically inactive, compound of CO_2 with hemoglobin. Actually, however, it only follows from Margaria's controls that the mean activity coefficient of $\overset{+}{Na}$ and $\overline{Cl}$ ions, i.e., $\sqrt{f_{Na} \times f_{Cl}}$, and of Na and of $H\overline{CO}_3$ ions, i.e., $\sqrt{f_{Na} \times f_{HCO_3}}$, is the same in hemoglobin solutions as in water: whereas what is really required is the effect of hemoglobin on the individual activity coefficients f_{Cl} and f_{HCO_3}. Experiments on the distribution of ions between hemoglobin solutions enclosed in a collodion membrane, and their dialysates, however, [v. infra, Adair (1928, 1934) Henriques (1928), (1929)] raise the possibility that the individual activity coefficients of $\overline{Cl}$ and $H\overline{CO}_3$ may be distinctly smaller in hemoglobin solutions than in water. If this were so it would reduce appreciably the amount of x-bound CO_2 as calculated by Margaria. From the physiological point of view Margaria's experiments are lacking in that they were not done exactly at physiological pH and temperature, nor was the hemoglobin studied in the reduced as well as the oxygenated conditions.

(ii) Depression of the freezing-point. Stadie and Sunderman (1931) report that the depression of the freezing-point produced by adding NaCl (concentrations 0.08 – 0.50 M) to solutions of hemoglobin (concentrations 13 – 40 grams per 100 grams water) is, within experimental error, the same as that produced by adding NaCl in the same proportion to water. From this they infer that hemoglobin is without effect on the osmotic activity coefficient of NaCl, and probably therefore would also be without effect on the osmotic activity coefficient of $NaHCO_3$. In experiments in which sodium hemoglobinate solution was equilibrated with CO_2 pressures ranging from 50 to 200 mm. Hg and pHs from c. 6.5 to c. 7.5, Stadie and O'Brien (1931) found an increased depression of the freezing point corresponding closely with that expected from the amount of CO_2 taken up, if the latter was fully osmotically active. They thus conclude, in opposition to Margaria, that no appreciable CO_2 bound directly to hemoglobin could be revealed osmometrically by freezing point technique. Apart from the possibility that the experiments of Stadie et al. were not accurate enough for the purpose (as suggested by Margaria, 1931) no explanation seems yet to have been given for the discrepancy between the conclusions of Margaria and of Stadie et al., both of which were derived from experiments which were thermodynamically of the same type, namely, osmometric. The same comments may be made on the researches of Stadie et al., as on those of Margaria: Stadie et al., however, recognise and discuss the relationship between the activity effects observed by them, and those observed by electrometric (including membrane equilibrium) methods: they point out that where the latter involve electrode determinations, there is an uncertainty as to whether the liquid junction potential between the protein solutions and the KCl bridge used to connect them with one or the other of the electrodes can be taken as zero, as is conventionally assumed. This uncertainty does not, however, arise in the membrane equilibrium experiments quoted above, wherein the distribution of ions Na, Cl and HCO_3 is measured by chemical analysis.

(iii) The total osmotic pressure of the interior of the red cells. Henriques (1933) has measured the distribution of water, protein, chloride, bound CO_2 and the various metals between the red cells and the serum at CO_2 pressures ranging from 10 to 470 mm. Hg, and temperature 37°C. He finds that the sum of the concentrations (expressed in millimols/litre of solution water) of metals, protein, chloride and bound CO_2, is about 5 per cent greater in the red cells than in the serum at pH 7.25, and that this excess gradually declines to zero as the pH is made more

acid or more alkaline. This should mean that the osmotic pressure of the interior of the red cell would be correspondingly greater than that of the serum. In view of the properties of the red cell membrane, Henriques contends that there can be no appreciable difference in osmotic pressure between the red cell and the serum: since he believes that the "non-electrolytes" are equally distributed between the serum and the red cells, he is forced to conclude that up to 5 per cent of the sum of the constituents measured by him is present in the red cell in some osmotically inactive form. Of this 5 per cent the main-fraction should be x-bound CO_2.

These data, unlike those previously considered, were obtained under conditions closely approximating to physiological. Henriques, however, assumes that the osmotic activity coefficients of the various ions is the same in the red cells as in the serum, and is in both cases independent of pH. Such an assumption is very dubious.[7] The seriousness of this point is shown (v. Ferguson and Roughton, 1934b)) by the fact that if the osmotic activity coefficient inside the red cell at pH 7.25 was only 5 per cent less than in the serum, the whole of Henriques' calculated effect at that pH would disappear. It should also be mentioned that similar determinations on red cells and serum by Van Slyke, Wu and Maclean (1923) failed to show any appreciable effect of the kind reported by Henriques, for the sum of the constituents in the cells agreed within experimental error with the sum of the constituents in the serum.

(iv) Adair measured the osmotic pressure of solutions of hemoglobin, which, after careful purification from all but minimal traces of base, had been equilibrated with CO_2 pressures at pH c.6.4, temperature 0°C. The osmotic pressure in such experiments is the sum of that due to the hemoglobin and that due to the unequal distribution of free bicarbonate ions between the hemoglobin solution and the outside solution. The fraction of the osmotic pressure calculated to be due to the free bicarbonate ions was only 0.6 to 0.8 of that to be expected from chemical estimation, in the solutions of the bound CO_2, if the latter was all present in the form of free bicarbonate ions.

The experimental conditions were certainly far from physiological but did reveal the same kind of behavior of CO_2 in hemoglobin solutions, as is found by most of the other methods.

The general conclusion, as to the rôle of x-bound CO_2 in CO_2 transport, from these different researches of an osmotic type, must be left to

[7] Furthermore, it is not clear whether possible changes in the degree of hydration of hemoglobin were taken into account.

the judgment of the reader, who has studied the cited papers of Margaria, Stadie et al., Henriques, and Adair closely. In the personal opinion of the reviewer, the osmometric evidence does on the whole, and in spite of its limitations, give qualitative support to the idea of a direct combination of CO_2 or of $H\overline{CO}_3$ or of both with hemoglobin in the pH range 7.0–8.0.

b. *Electrometric methods.* (i) The pK_1' of the Henderson-Hasselbalch equation in hemoglobin solutions.

The principle of the method adopted by Margaria and Green (1933) may be stated as follows:

Let K_1' = the apparent first ionisation constant of carbonic acid i.e.,

$$K_1' ([CO_2] + [H_2CO_3]) = [a_H] [a_{HCO_3}]$$

where a_H, a_{HCO_3} are the respective activities of the hydrogen and bicarbonate ions, a_{HCO_3} being equal to $f_{HCO_3} [H\overline{CO}_3]$. Since at equilibrium $[CO_2]$ = about 700 $[H_2CO_3]$.

The equation may be written with sufficient accuracy

$$K_1' [CO_2] = [a_H] f_{HCO_3} [H\overline{CO}_3]$$

Taking logarithms of each side and transposing

$$pa_H = pK_1' + \log f_{HCO_3} + \log \frac{[H\overline{CO}_3]}{[CO_2]} \qquad (10)$$

where $pK_1' = -\log_{10} K_1'$.

This is the modern form of the Henderson-Hasselbalch equation. Of the quantities in this equation pa_H was measured electrometrically (by the glass electrode in the case of Margaria and Green's experiments, and by the hydrogen electrode in Stadie and Hawes' work, 1928). The value of $[CO_2]$ was obtained from analysis of the gas phase with which the hemoglobin was equilibrated, and the solubility constant of CO_2.

p K_1' is given by experiments on solutions of CO_2 and of bicarbonate containing no protein. The value at 38°C. = 6.33.

log f_{HCO_3} was taken = $-0.5\sqrt{\mu}$, μ being the ionic strength.

This equation for f_{HCO_3} has been verified in inorganic solutions of sodium bicarbonate ± sodium chloride up to ionic strengths of 0.4 M by Hastings and Sendroy (1925), Stadie and Hawes (1928), and Margaria and Green (1933). Margaria and Green assumed that it was applicable in hemoglobin solutions, the contribution of the hemoglobin

to the ionic strength being taken as equal to that by sodium ions of concentration electrochemically equivalent to that of the hemoglobin. Thus, e.g., in experiment 7 of their table 2:

The solution contained Hb = 0.016 M, $\overset{+}{Na}$ = 0.032 M, $\overline{Cl}$ = 0.0053 M. The Hb is electrochemically equivalent to

$$[\overset{+}{Na}] = 0.0\,32 - 0.0053 = 0.0267\ M$$

The ionic strength then $= \frac{1}{2}\Sigma c\nu^2 = \frac{1}{2}\,(0.032 \times 1^2 + 0.0053 \times 1^2 + 0.0267 \times 1^2) = 0.032$. Knowing the pa_H, pK_1' and log f_{HCO_3} the value of $[H\overline{CO_3}]$ is calculated from the Henderson-Hasselbalch equation

The x-bound CO_2 then = total $[CO_2]$ as estimated by acid-vacuum extraction − [dissolved CO_2] − $[H\overline{CO_3}]$ as above calculated.

On this basis, Margaria and Green obtained values ranging from 0.0 to 0.009 M x-bound CO_2 in purified hemoglobin solutions, oxygenated and reduced, CO_2 pressures ranging from 10 to 800 mm. Hg pH 6.0 − 8.0, temperature 38°C. The amount of x-bound CO_2 in reduced hemoglobin solutions was, on the average, distinctly greater than in oxygenated hemoglobin solutions under otherwise parallel conditions. On the basis of their experiments they compute that of the total-bound CO_2 in the red cells one-third to one-quarter is present in the x-bound form, although only 5 or 10 per cent of the total in the blood is in that form. As regards the respiratory cycle they compute that three-quarters of the change is bound CO_2 in the red cells, and one-third of the total change in the blood between the arterial and venous condition is due to changes in x-bound CO_2.

An uncertain feature in this research is the assumption that as regards its contribution to the ionic strength, μ, hemoglobin functions as a univalent component. For this, as already pointed out, there is no convincing evidence: thus, as mentioned above, some of the osmotic evidence would suggest that hemoglobin need not be considered as regards its contribution to the ionic strength, whilst in studies on the effect of salts on the solubility of oxyhemoglobin, on the other hand, Cohn and Prentiss (1927) and Green (1931) find that hemoglobin behaves, from the activity and ionic strength point of view, as though it were an electrolyte of valency not less than 2. If the latter were assumed to be the case in Margaria and Green's experiments, the calculated amount of x-bound CO_2 would be much decreased, especially at pHs > 7.0 and relatively low total bound CO_2.

Stadie and Hawes (1928) previously made an extensive series of

observations on pK_1' in solution of varying concentrations of carbon monoxide hemoglobin, reduced hemoglobin, methemoglobin, cyanhemoglobin and nitric oxide hemoglobin at varying ionic strength. They found results of the same kind as Margaria and Green, but attributed them exclusively to specific effects of hemoglobin on the activity coefficient of the bicarbonate ion and not to x-bound CO_2. The effect of reduced-, met-, and cyan-hemoglobin was, however, found to be much greater than that of carbon monoxide- or nitric oxide- hemoglobin. This they suggest tentatively may be due to the latter group of derivatives of hemoglobin causing a smaller increase in the di-electric constant of water than the former group, with a consequent smaller effect on the inter-ionic forces. So far as the reviewer is aware this has not yet been proved by experiment and seems rather unlikely. A more probable explanation of Stadie and Hawes' results is that the reduced hemoglobin group forms x-bound CO_2 more readily than the carbon monoxide hemoglobin group: this is strongly supported by the fact that, as already shown, reduced hemoglobin forms the carbamino fraction of the x-bound CO_2 more readily than oxyhemoglobin, and since, as regards combination with CO_2, oxyhemoglobin and carbon monoxide hemoglobin appear to behave in an identical way, the latter should also form the carbamino fraction of the x-bound CO_2 less readily than reduced hemoglobin.

Dill and Forbes (private communication) point out that the calculation of the x-bound CO_2, with the aid of the Henderson-Hasselbalch equation, is most convincing when both the dissolved CO_2, the total bound CO_2 and the ionic strength are small, for in such cases the x-bound CO_2/total CO_2 tends to reach its highest value, and the uncertainties both as to the activity coefficient corrections, and as to the solubility coefficient of CO_2 in hemoglobin solutions are of minimal effect. Thus, e.g., in the first line of experiment 4 in table 2 of Margaria and Green's paper (1933):—$pCO_2 = 10.1$ mm., dissolved $CO_2 = 0.0003$ M, total bound $CO_2 = 0.0014$ M, ionic strength $= 0.005$ M, calculated value of x-bound $CO_2 = 0.00067$ M. The value of f_{HCO_3} would have to be 50 per cent lower than that assumed, if the computed x-bound CO_2 is to be reduced to zero.

Dill and Forbes have obtained even more striking instances of a similar kind, and so also has Willmer (1934) in his recent work on the blood of fishes which inhabit the acid waters of tropical swamps. This observer has found that the pH of these bloods, in many cases, lies in the range pH 5.5 to 6.5 and that the observed pH when the bloods are equilibrated with $pCO_2 = 25$ mm. Hg may be nearly 1.0 pH unit more acid

than the value expected from the Henderson-Hasselbalch equation if all the bound CO_2 is in the form of free $H\overline{CO}_3$. This discrepancy would require an activity coefficient of only 0.1 for the bicarbonate ion, if it is to be explained without postulating the existence of x-bound CO_2. So low a value of the activity coefficient would seem extremely improbable. It would be interesting to have data on mammalian hemoglobin under similar conditions of CO_2 pressure and pH.

As regards mammalian whole blood, the situation has been dealt with in the earlier work of Van Slyke, Hastings, Murray and Sendroy (1925). The concentrations of all the different ions were respectively determined both in the serum and in the red cells, and the values for f_{HCO_3} calculated therefrom by the Debye-Hückel equation, the protein being assumed to function as a univalent component. For the serum the value of f_{HCO_3} was calculated to be 0.625. Substitution of this value for f_{HCO_3} in equation (10), together with the values for a_H, total CO_2 and dissslved CO_2 found in determinations on serum equilibrated with various CO_2 pressure in the physiological range of pH and temperature, led to values for $[H\overline{CO}_3]$, which agree within experimental error with the difference between the total CO_2 of the serum and the dissolved CO_2. There would thus not appear to be any x-bound CO_2 in serum, under physiological conditions, unless the contribution of the protein to the ionic strength (in the Debye-Hückel equation) has been *over-estimated.* From the figures of Van Slyke et al., the reviewer calculates that if to take the extreme case the serum proteins make zero contribution to the ionic strength, the x-bound CO_2 would only amount to about 2.0 per cent of the total bound CO_2 in the serum.

Similar calculations and experiments with the red cells gave a value for f_{HCO_3}, which is practically the same as the serum value, namely, 0.62. This, when substituted in equation (10), led on the other hand to values for $[H\overline{CO}]_3$, which are only about two-thirds as large as the difference between the total CO_2 of the red cells and the dissolved CO_2 therein. These results are thus similar to those of Margaria and Green, and of Stadie and Hawes, and the same comments apply as in those cases.

(ii) Membrane potential and distribution experiments. The main experiments of this kind have been carried out by Henriques (1928, 1929), Adair (1928, 1934) and Van Slyke, Hastings, Murray and Sendroy (1925), and have already been briefly mentioned. Henriques' experiments were carried out as follows:

Washed red cells were laked with saponin, the "detritus" removed by centrifuging, and the concentrated hemoglobin solution (c. 30 per cent)

then reduced. The latter was then treated with $NaHCO_3$ and NaCl (0.03–0.10 M), and after equilibration with pressures of CO_2 ranging from 25 to 600 mm. Hg, was dialysed in collodion sacs, under sterile conditions and without contact with air, against an outside fluid containing suitable concentrations of $NaHCO_3$ and NaCl. After 12 hours' dialysis at 18°C. equilibrium was reached. The following measurements were then made (α) the difference of electrical potential, E, between the inside and outside fluids, i.e., the Donnan membrane potential (β) pa_H of the outside fluid (by indicators or the quinhydrone electrode) (γ) the pa_H of the inside fluid (by hydrogen electrode) (δ) the total CO_2 of the inside and of the outside fluid (Van Slyke gasometric method) whence could be calculated the total bound CO_2 in each case. (ϵ) the chloride concentration of the inside and outside fluids (by chemical analysis). At equilibrium the following relations must thermodynamically hold good:

$$E = \frac{RT}{F} \log \frac{a'_H}{a_H} = \frac{RT}{F} \log \frac{a_{Cl}}{a'_{Cl}} = \frac{RT}{F} \log \frac{a_{HCO_3}}{a'_{HCO_3}} \qquad (11)$$

or

$$\frac{a'_{H'}}{a_H} = \frac{a_{Cl}}{a'_{Cl}} = \frac{a_{HCO_3}}{a'_{HCO_3}} \qquad (12)$$

F is the electric charge on 1 gram ion, symbols with dashes refer to the activities of the ions of the inside liquid, symbols without dashes refer to the outside fluid. Measurements (α), (β), (γ) confirmed that the equation $E = \frac{RT}{F} \log \frac{a'_{H'}}{a_H}$ did hold good:[8] this proves that equilibrium had been reached. From measurements (δ) and (ϵ) it appeared that [total chloride]/[total chloride]′ and the [total bound CO_2]/[total bound CO_2′]′ were both less than a'_H/a_H, the former by 10 to 20 per cent the latter by 20 to 30 per cent, i.e., there was more chloride and bound CO_2 in the inside hemoglobin solution than was expected from the difference of pa_H between the inner and outer solutions.

In order to reconcile these findings with equation (12), it is necessary to make use of one or of both of the already mentioned suggestions, namely, that *a*, hemoglobin has a specific lowering effect on the activity

[8] There are some elements of uncertainty in the data quoted by Henriques, amongst which may be mentioned that the pa_H values given for the dialysates often disagree considerably from the values of pa_H to be anticipated from the CO_2 pressures and CO_2 contents of the dialysate.

coefficient of the $\overline{Cl}$ and $H\overline{CO_3}$ ions, the latter being more affected than the former. *b*. Both $\overline{Cl}$ and $H\overline{CO_3}$ (and/or CO_2) combine directly with the hemoglobin.

In the experiments of Adair et al., (1928, 1934a, b) purified solutions of carbon monoxide hemoglobin were dialysed at 0°C., and pH range 6.0 to 8.0, against outside solutions containing several or all of the ions $\overset{+}{Na}$, $\overset{+}{NH_4}$, $\overline{Cl}$, $\overline{H_2PO_4}$, $\overline{\overline{HPO_4}}$, and of course H and $\overline{OH}$. In addition to the estimations made in Henriques' experiments the concentrations of Na, NH_4 and total phosphate in the hemoglobin solution and in the dialysate at equilibrium were determined chemically. Adair et al. found experimentally that

$$E\begin{pmatrix}\text{membrane}\\ \text{potential}\end{pmatrix} = \frac{RT}{F}\log\frac{[Na]'}{[Na]} = \frac{RT}{F}\log\frac{[NH_4]'}{[NH_4]} \text{ to within about } 5 \text{ per cent}$$

the liquid junctions potentials being assumed as usual to be zero. If this conventional assumption is, in this instance, correct, then it follows from equation (11) that the activity of both the cations, $\overset{+}{Na}$ and $\overset{+}{NH_4}$, is but slightly affected by the presence of the hemoglobin over the pH range 6.0 to 7.6. Presumably then there is no marked effect of hemoglobin on the activity coefficients of $\overset{+}{Na}$ or $\overset{+}{NH_4}$, nor do either of these ions combine appreciably with hemoglobin under the conditions of the experiment. In the case of the anions, however, it was found that both [Cl]/[Cl′] and [phosphate]/[phosphate′] were lower than [Na′]/[Na], the former by 10 to 20 per cent, and the latter by 20 to 50 per cent. In general the discrepancies were greatest near the iso-electric point, and decreased as the pH was made more alkaline. This last point, Adair argues, is much easier to reconcile with the existence of direct compounds of the anions with hemoglobin of the type $^{+}Pr^{-} + Na^{+} + \overline{Cl} = [\overline{Cl}^{+}\ Pr^{-}] + Na^{+}$, where $^{+}Pr^{-}$ represents the iso-electric protein according to the modern Zwitterion theory, than to explain by effects of hemoglobin on the inter-ionic forces. Adair also adduces other arguments in favour of the existence of such compounds, of which perhaps the most striking comes from his experiments on the equilibrium of carbon monoxide hemoglobin crystals and their mother liquor in ammonium phosphate buffer solutions near the iso-electric point of hemoglobin. There is a marked excess of both $\overset{+}{NH_4}$ and phosphate in the crystals over that found in the mother liquor. This is easy to explain if chemical compounds are formed, but not otherwise, e.g., by "inter-ionic effects" or

by Donnan equilibrium ionic distribution effects between the interior of the crystals and the mother liquor.

Adair has also obtained data (not yet published) on the distribution of bound CO_2 between hemoglobin solutions and dialysates. The discrepancies for bound CO_2 are greater than for Cl, and are indeed nearly the same as for phosphate, but do not decline so much at alkaline pH as in the latter case. This is to be expected if the discrepancy is due to x-bound CO_2, for this includes not only $H\overline{CO}_3$ bound to hemoglobin, after the same fashion as $\overline{Cl}$ or phosphate are supposed to be bound, but also CO_2 bound to hemoglobin in the carbamino form, which latter increases when the pH is made more alkaline. As regards the distribution of bound CO_2 between hemoglobin crystals and mother liquor, a short preliminary note has recently been published by Van Slyke, Dillon and Hiller (1933) on results obtained with the readily crystallisable hemoglobin of the rat. These authors report an excess of bound CO_2 in the crystals over that in the mother liquor, the extent of the excess depending on the CO_2 pressure and total CO_2 content of the mother liquor. Further work on these lines is very desirable.

That the results obtained for hemoglobin solutions separated from their dialysates by collodion membranes, also hold good for hemoglobin in the red cell is shown by the earlier work of Van Slyke, Hastings, Murray and Sendroy (1925). In this research it was found that the distribution of $\overset{+}{H}$, $\overline{Cl}$ and total bound CO_2 between the serum and the red cells could be summarised by the equation

$$\frac{[a_H]_c}{[a_H]_s} = 1.3\,\frac{[Cl]_s}{[Cl]_c} = 1.6\,\frac{[\text{total bound } CO_2]_s}{[\text{total bound } CO_2]_c} \qquad (13)$$

where c suffixes refer to concentrations in the red cells, s suffixes to the serum. These results are thus of the same order of magnitude as those of Henriques and Adair.

(iii) Comparisons of the titration curve of hemoglobin with HCl and with CO_2 respectively. These tests have been carried out by Van Slyke et al. (1922, 1924), Hastings, Sendroy, Murray and Heidelberger (1924) and Stadie and O'Brien (1931). For the titration curve with HCl, crystals of purified hemoglobin were dissolved in standard amounts of NaOH or KOH, and titrated back with HCl, the pH after each HCl addition being measured with the hydrogen electrode. The range covered was about pH 8.5 to 6.0. The base, "BHb," electrochemically equivalent to the hemoglobin was calculated by the equation:

$$[BHb] = [\text{total alkali added}] - [HCl\ \text{added}] \qquad (14)$$

it being assumed for the moment that no Cl or HCl was bound to the hemoglobin directly.

For the titration curve with CO_2, a similar solution of hemoglobin in standard alkali was equilibrated with CO_2 at known pressure, the quantitative relationships being chosen so as to resemble those occurring in blood in vivo. The total CO_2 was measured gasometrically and the pH (in the more recent experiments) electrometrically. In this case BHb was calculated by the equation

$$[BHb] = [\text{total alkali added}] - [\text{total bound } CO_2] \qquad (15)$$

it again being assumed for the moment that the whole of the bound CO_2 is in the form of free $H\overline{CO_3}$ ions.

By determinations and calculations on this basis, Van Slyke et al. found exactly the same proportions of alkali electrochemically equivalent to hemoglobin at the same pH levels, whether the titration was made with HCl or CO_2, and whether the hemoglobin was reduced or combined with carbon monoxide (carbon monoxide hemoglobin has the same alkali binding power as oxyhemoglobin). From this they conclude that either the total chloride and total CO_2 remain completely free, i.e., do not combine appreciably with the hemoglobin, or else that chloride and CO_2 are each bound to the same extent at each pH. The latter possibility was considered by Hawkins and Van Slyke (1930) to be too far outside the limits of probability to merit consideration.

The results of Van Slyke et al. with carbon monoxide hemoglobin were confirmed by Stadie and O'Brien (1931): in regard to reduced hemoglobin, however, inspection of sections III b and IV b in table 1 of their paper show that the titration curves with HCl and CO_2 are only reconciled by assuming, on the average, about 0.0015 M CO_2 bound directly to the hemoglobin (as contrasted with — 0.0002 M CO_2 so bound in the case of carbon monoxide hemoglobin). Such a difference between reduced hemoglobin and carbon monoxide hemoglobin would be physiologically significant (v. previous section on physiological rôle of carbamino compounds) if it were definitely established but may possibly be not outside the limits of experimental error of Stadie and O'Brien's methods.

Even if it be granted that the titration curves of hemoglobin with HCl and CO_2 are identical both for carbon monoxide hemoglobin and for reduced hemoglobin, there are two objections to Hawkins and Van

Slyke's opinion that this result constitutes evidence against the existence of quantitatively important amounts of CO_2 bound directly to hemoglobin.

(α) (due to Henriques, Margaria, and Roughton independently). The argument of Hawkins and Van Slyke only holds good if CO_2, when combined directly with hemoglobin, is not free to balance an equivalent amount of base. If, however, CO_2 is bound to hemoglobin in the carbamino form, i.e.,

$$HbNH_2 + CO_2 \rightarrow HbNHCOOH \rightarrow HbNH\overline{COO} + \overset{+}{H}$$

each molecule of CO_2 so bound will give rise to a new COOH group in hemoglobin. According to Faurholt's views upon the strength of acids of the carbamino types, such COOH groups would tend to ionise largely at pHs in the neighbourhood of 7.0. CO_2 molecules bound to hemoglobin in the carbamino form might therefore lead to the production in the hemoglobin of nearly an equivalent number of negatively charged $-\overline{COO}$ groups, which latter would of course neutralise an equivalent amount of base. This type of compound of CO_2 with hemoglobin might thus easily escape detection by the titration curve criterion of Van Slyke, especially if it was only present in amounts, which were absolutely, but not physiologically small. Such behaviour is just what has been recorded of the carbamino type of compound of CO_2 with hemoglobin discussed in the previous section.

(β) Of the total CO_2 bound to hemoglobin, a part may consist, not of carbamino bound CO_2, but of bound $H\overline{CO_3}$ ions. In view of the results discussed in the previous sub-section on membrane equilibria, it would be rather rash to conclude, with Van Slyke and Hawkins, that $\overline{Cl}$ and $H\overline{CO_3}$ could not bind with hemoglobin on the alkaline side of the isoelectric point in roughly equal amounts.

On these grounds it has been suggested that the identity of the titration curves of hemoglobin with HCl and CO_2 does not at present constitute decisive evidence either for or against the existence of x-bound CO_2.

CONCLUSIONS AS TO X-BOUND CO_2, CARBAMINO-BOUND CO_2 AND Y-BOUND CO_2 IN BLOOD TRANSPORT OF CO_2. The investigations of Margaria (1931) and Van Slyke et al. (1925) on the total x-bound CO_2 of the serum, and of Roughton on the carbamino-bound CO_2 fraction, all failed to reveal significant traces of any of these compounds in the serum (v. p. 273 of this review). For the present, then, the problem of the rôle of

these compounds in CO_2-transport only arises in regard to events in the red cells.

That one particular fraction of the x-bound CO_2, namely, the carbamino fraction, does exist in Hb solutions and in the red blood corpuscles under physiological conditions, of pH and temperature, seems to be established. This compound may be responsible for as much as 20 per cent of the CO_2 transport by the blood during the respiratory cycle.[9]

Conclusions as to the existence and amount of y-bound CO_2 should, in view of the difficulties already referred to in the determination of x-bound CO_2, be more tentative, though for the present, positive. Thus,

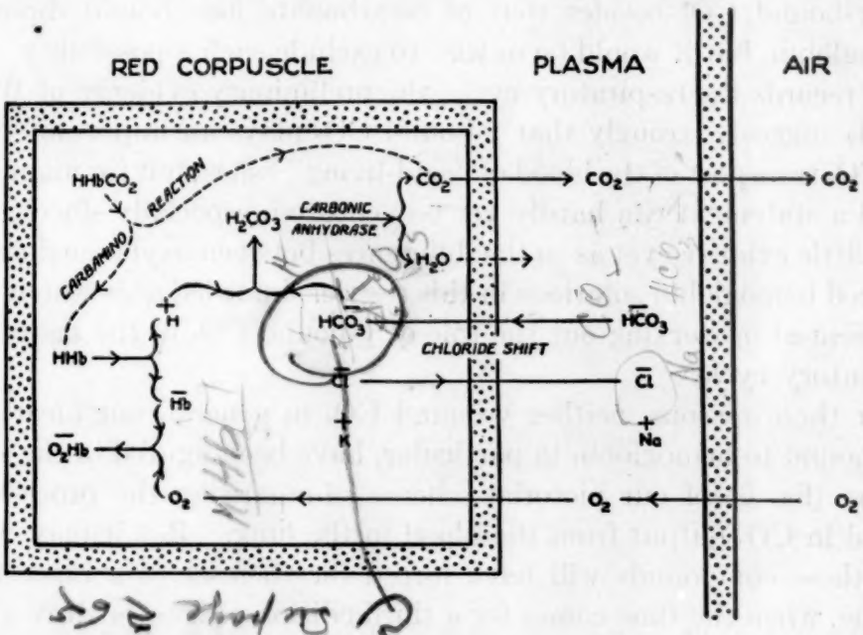

Fig. 6. Schematic survey of main processes occurring with the blood, during CO_2 output, according to present viewpoint (Cp. fig. 1).

as Ferguson and Roughton (1934b) point out, the quantitative results obtained in most of the methods described on pp. 265, 266 of this review, are most simply interpreted on the view that, under physiological conditions, there is definitely more x-bound CO_2 than carbamino-bound CO_2 in hemoglobin solutions, and presumably therefore also in the red blood

[9] Note added in proof:

In a recent number of Science (February 22, 1935) Stadie has criticised the inferences drawn by Meldrum and Roughton, from their experiments on the rate of uptake of CO_2 by cyanide blood (v, pp. 270–273), as to the rôle of carbamino-bound CO_2 under normal physiological conditions. In the writer's opinion, Stadie's criticism is invalid for reasons to be given in a forthcoming article in Science.

corpuscles. Ferguson and Roughton also report preliminary experiments with Adair on the *same* hemoglobin solution, using the membrane distribution method for x-bound CO_2 and the barium precipitation method for carbamino bound CO_2—these results too fall into line with this view.

Experiments with other ions than bicarbonate, i.e., with chloride and phosphate, are likewise most simply explained by the direct combination of these anions with hemoglobin even on the alkaline side of its iso-electric point—if other simple anions combine, so also should bicarbonate. There is no indication at present of any other constituent of the y-bound CO_2 besides that of bicarbonate ions bound directly to hemoglobin, but it would be unwise to exclude such a possibility.

As regards the respiratory cycle, the preliminary evidence of Willmer (1934) suggests strongly that y-bound CO_2 plays an important part in the CO_2 transport of the blood of "acid-living" fishes; but for mammalian blood a statement can hardly yet be ventured, especially since there is very little evidence yet as to the differences between oxyhemoglobin and reduced hemoglobin solutions in this respect, knowledge of which would be essential in working out the rôle of y-bound CO_2 in the mammalian respiratory cycle.

For these reasons, neither y-bound CO_2 in general, nor bicarbonate ions bound to hemoglobin in particular, have been figured in the second edition (fig. 6) of our pictorial scheme summarising the processes involved in CO_2 output from the blood in the lung. But it may well be that these compounds will have forced for themselves a place in the scheme, when the time comes for a third edition of it to be drawn up.

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